NITROBLUE TETRAZOLIUM TEST AS AN ASSAY OF NEUTROPHIL FUNCTION IN DIABETES MELLITUS

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CERTIFICATE

This is to certify that this dissertation entitled **"NITROBLUE TETRAZOLIUM TEST AS AN ASSAY OF NEUTROPHIL FUNCTION IN DIABETES MELLITUS"** is a bonafide work done by **Dr. A.P. JONATHAN ARNOLD,** in partial fulfillment of the requirements of The TAMIL NADU DR.M.G.R. MEDICAL UNIVERSITY, Chennai for the award of **M.D. Pathology Degree.**

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I declare that this dissertation entitled **"NITROBLUE TETRAZOLIUM TEST AS AN ASSAY OF NEUTROPHIL FUNCTION IN DIABETES MELLITUS"** has been done by me under the guidance and supervision of **Prof. Dr. M.P.KANCHANA, M.D.** It is submitted in partial fulfillment of the requirements for the award of the M.D., Pathology degree by The Tamilnadu **Dr. M.G.R. Medical University**, Chennai. This has not been submitted by me for the award of any degree or diploma from any other University.

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ABBREVIATIONS & STATISTICAL FORMULAE

ANC	–	Absolute Neutrophil count
AGEs	–	Advanced Glycosylation End products
CGD	–	Chronic Granulomatous Disease
C.I	–	Confidence Interval (Mean +/- 2SE) i.e., 95% of the means from similar samples drawn from the same population will have their value within the limits of two SE.
DLC	–	Differential Leucocyte Count
EDTA	-	Ethylene Diamine Tetraacetic Acid
ESR	–	Erythrocyte Sedimentation Rate
FBS	–	Fasting Blood Sugar
FMLP	–	Formyl Methionyl – leucyl - phenylalanine
n	–	number of cases
n_1	-	number of cases (1st category)
n_2	-	number of cases (2nd category)
NADPH phosphate	–	Nicotinamide adenine dinucleotide (reduced)
NAP	–	Neutrophil Alkaline Phosphatase
NBT	–	Nitroblue Tetrazolium
P	-	Probability
P<0.05	–	Statistically significant at 5% level
P>0.05	-	Not statistically significant at 5% level
p_1	=	proportion of cases (1st category)
p_2	=	proportion of cases (2nd category)
q_1	=	$1-p_1$
q_2	=	$1-p_2$
σ (sigma)	–	Standard Deviation
σ_1	–	Standard Deviation of the 1st category
σ_2	–	Standard Deviation of the 2nd category

SE	–	Standard Error of the mean (Standard Deviation / $\sqrt{n}$)
Standard Error of Difference Between two Means	=	$\sqrt{[\sigma_1^2/n_1]} + \sqrt{[\sigma_2^2/n_2]}$
Standard error of difference between two proportions	=	$\sqrt{[p_1q_1/n_1 + p_2q_2/n_2]}$
UTI	–	Urinary Tract Infection
Z score for standard error of difference between two means	=	$\{Mean_1 - Mean_2\}$/standard error of difference between two means
Z score for standard error of difference between two proportions	=	p_1-p_2/standard error of difference between two proportions

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INTRODUCTION

Nitroblue tetrazolium test is one of the simplest and cost-effective methods in the assessment of neutrophil bactericidal function. This has also been used as a method of early diagnosis in bacterial infection.

Neutrophilic leucocytosis is considered as an indirect evidence of acute inflammation and acute infections though it is seen in several other conditions. To combat any infectious process, apart from the rise in leucocyte counts, a normal neutrophil function is also essential.

Patients with a neutrophilic response on exposure to infectious agents need not necessarily possess normal neutrophil functions like chemotaxis, phagocytosis, killing and degradation.

Diabetes mellitus is a common disease in which neutrophil functions are altered [1,2,3]

A battery of tests are available to assess neutrophil functions which include NBT test, flow cytometry, spectrophotometric assays, chemotaxis assays, superoxide assays and immunoblotting[4] which though accurate require expertise and monetary consideration while the NBT test is inexpensive and technically less demanding .

This prospective study focuses on the utility of NBT test to study neutrophil function in diabetics and to reiterate its role as a supplement in the diagnosis of bacterial infections.

AIMS AND OBJECTIVES

1. To describe about the NBT test, its relation to bactericidal function of neutrophils and its scoring systems.
2. To compare the NBT scores in non-diabetics with and without bacterial infection.
3. To compare the NBT scores in diabetics with and without bacterial infection and show its usefulness in the assessment of response to infection in the diabetic population.
4. To compare the difference in NBT scores between non-diabetics and diabetics without infection.
5. To compare the difference in NBT scores between non-diabetics with infection and diabetics with infection and analyze if it could be related to the defective bactericidal function in diabetic patients.
6. To observe the relationship between NBT score and absolute neutrophil count.

REVIEW OF LITERATURE

Peripheral blood leucocytes include neutrophils, eosinophils, basophils, lymphocytes and monocytes. The mature neutrophil measures 12-15µ in diameter. The cytoplasm is acidophilic with fine granules. The nucleus has clumped chromatin and has 2-5 distinct lobes separated by filaments which are narrow strands of dense heterochromatin. Band cell is a granulocytic cell with a curved or coiled band shaped nucleus. Small number of band cells are seen in healthy subjects.

Neutrophilia: Causes are numerous and common ones include acute infections, inflammation, intoxication, corticosteroid therapy, pregnancy and acute blood loss, among others.

Neutrophilic granules: The neutrophilic granules[4] serve as reservoirs for digestive and hydrolytic enzymes. They are classified primary, secondary and tertiary granules.

- *Primary granules* (azurophilic): The primary granules contain myeloperoxidase, neutral proteases which include elastase and cathepsins, protease inhibitors, acid hydrolases, acid phosphatase α mannosidase, N-acetyl glucosaminidase and cationic proteins. Azurophil granule proteins possess microbicidal activity and have a role in tissue destruction during inflammatory reaction. Myeloperoxidase reacts with

H_2O_2 and a halide to produce hypochlorous acid, a potent oxidant, believed to significantly contribute to the killing of microorganisms.

- *Secondary granules* (specific): Proteins present in these granules include lysozyme, collagenase, vitamin B_{12} binding protein, heparanase and lactoferrin. Lysozyme hydrolyzes cell wall proteoglycan of some bacteria. Lactoferrin is necessary for hydroxyl radical formation and can influence the function of lysozyme to kill gram-negative bacteria. Heparanase helps in the extravasation of neutrophils. These granules' membrane possess cytochrome b which regulates respiratory burst.

- *Tertiary granules*: These contain gelatinase, FMLP receptor, cytochrome b, CD11b, CD18 and CD16 which are essential for cell adhesion.

Other granules are phosphosomes and secretory granules.

Functions Of Neutrophils:

The neutrophils protect the host against pyogenic infections. The cellular events in acute inflammation include mobilization of neutrophils from marrow stores, adhesion and extravasation of the leucocytes, chemotaxis, leucocyte activation, phagocytosis, phagolysosome formation, respiratory burst and ultimately killing and degradation of the organisms[4,5].

Bactericidal Mechanisms of neutrophils:

This includes oxidative and non-oxidative mechanisms of which the former is more important. Phagocytosis stimulates a burst in O_2 consumption (respiratory burst), glycogenolysis, increased glucose oxidation via HMP shunt and production of reactive oxygen metabolites by the granules of activated neutrophils. The reactive oxygen species include superoxide anion(O_2^-), hydrogen peroxide(H_2O_2), hydroxyl radicals($OH^{\bullet}$**),** hypochlorous acid (HOCl) and singlet oxygen. These reactive intermediates are generated by the NADPH oxidase, located on the plasma membrane which reduces molecular oxygen to O_2^-. The oxidase is quiescent in resting neutrophils and is stimulated following neutrophil activation.

Synthesis of reactive oxygen intermediates:

$$NADPH + 2O_2 \xrightarrow{\text{NADPH oxidase}} NADP + 2\ O2\text{-} + H+$$

$$O_2^- + O_2^- + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2$$

$$H_20_2 + Cl^- \xrightarrow{\text{Myeloperoxidase}} H_20 + OCl^-$$

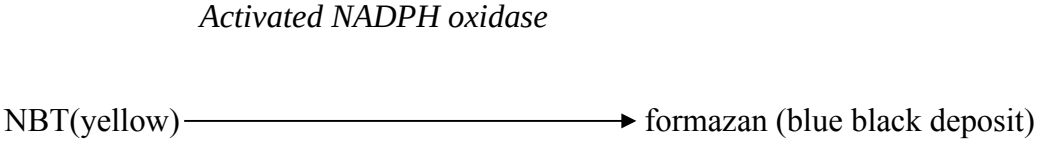
Neutrophil Function Tests

1) Nitroblue tetrazolium test- assesses the respiratory burst activity.
2) Flow cytometry using dihydrorhodamine 123 fluorescence (DHF)- measures respiratory burst leading to the production of reactive oxygen species.
3) Flow cytometry to measure CD11/CD18 surface glycoproteins on neutrophils.
4) Flow cytometry to measure L-selectin on neutrophils.
5) Spectrophotometric assay to measure the cytochrome B content in an extract of detergent – disrupted neutrophils.
6) Superoxide assays like chemiluminescence tests.
7) Chemotaxis assays like polarization assay (studies the response of neutrophils to chemotactic factors) and the Rebuck skin window test.
8) Quantitative ingestion assays (patient & control sera as opsonins).
9) Myeloperoxidase staining and estimation.
10) Immunoblotting- estimates cytochrome B subunit and cytosol oxidase component to differentiate X-linked from autosomal recessive forms of chronic granulomatous disease.[4]

Principle Of The NBT Reaction:

Under normal conditions, the enzyme NADPH oxidase in neutrophils is in an inactive state. During bacterial infections, phagocytosis of the microbes occur resulting in fusion of lysosome with phagosome and activation of NADPH oxidase. This enzyme is essential as mentioned earlier for production of oxygen derived free radicals.

Nitroblue tetrazolium is a colorless or yellow dye, which can penetrate the neutrophil cell membrane. If neutrophils are stimulated, NADPH oxidase converts the dye in the phagolysosome into blue black deposits called as *formazan* which can be observed under the microscope. In unstimulated conditions (like non-infectious states), most of the neutrophil oxidase is inactive and hence formazan formation on addition of NBT dye is less.[6]



Hence, the formation of formazan, is an indirect evidence to the degree of respiratory burst activity of neutrophils.

Biological basis for increased NBT in infection:

McCall *et al*[7], conducted NBT test along with other bactericidal indices like oxygen utilization and hexose monophosphate shunt activity. They observed significant elevations in bactericidal indices above that of controls, in all patients with elevated NBT scores. Thus it proved that increased

bactericidal capacity of neutrophils was the primary cause of increased NBT reduction.

History of the NBT test:

The origin of NBT test dates back to 1967, when Baehner and Nathan[8] showed that while a small proportion of the neutrophils of normal subjects could invitro reduce the soluble dye to insoluble formazan precipitate, leucocytes from subjects with chronic granulomatous disease(CGD) were unable to effect this reaction, thus providing a sensitive diagnostic test for the diagnosis of the later condition.

In 1968 , Park, Fikrig and Smithwick[9] described NBT as a rapid aid to the diagnosis of bacterial infection in children. Enhanced NBT reduction was reported by Feigin *et al*[10] (in systemic bacterial infections), Anderson *et al*[11](malaria),Park *et al*[9](systemic mycosis), Humbert *et al*[12](new born infants) and in scarlet fever.[13]

Gordon *et al*[6] , compared the NBT reduction scores between healthy volunteers and those with bacterial and viral infection and found significantly higher scores in NBT scores in bacterial infections compared to the latter.

Adnan *et al* [14] in 1972 showed that when skin of hamsters were experimentally infected with streptococci & staphylocci strains, an increase in percentage and absolute number of NBT positive neutrophils occurred.

In cases with doubtful diagnosis of pulmonary thromboembolism and lobar pneumonia existed, the role of NBT was proved by Rowan *et al*[15] in

1974, thus showing that NBT is useful to distinguish infective from non-infective lesions. Similar results were obtained by Hellum KB *et al*[16] in 1977.

An important application of NBT in rapid screening of neonatal infections/sepsis where a high NBT score correlated with the presence of infection was demonstrated by Dalens *et al*[17] in 1981.

Though NBT test was useful in distinguishing bacterial from viral infections, Trojan *et al*[18] observed that both early cases of bacterial meningitis and viral encephalitis had a low NBT score in their blood samples. In contrary Kolmel *et al*[19], studying granulocytes from CSF samples, observed higher NBT scores among bacterial meningitis patients compared with non-bacterial meningitis.

The effects of antibacterial drugs on NBT score was studied by Hellum KB *et al*[20] in 1977 , who observed that there was a higher mean NBT score among the patients who had not received any antibacterial therapy compared with those who had received the treatment. Dalens *et al*[17] showed that first few days of antibacterial treatment does not alter the NBT reduction.

Miller RM *et al*[21] in 1976,observed enhanced NBT reduction in febrile patients with bacterial infection compared to those febrile due to non-bacterial and non-infectious conditions.

Infection And Diabetes Mellitus:

Some infections are more frequent in those with diabetes and some are more aggressive in the diabetic host.[22] There are multiple defects in immunity in diabetes which explains the susceptibility to infection including impaired

neutrophil function.[1-3] There are also other secondary causes such as frequent hospitalization, delayed wound healing and chronic renal failure.

Urinary Tract Infection(UTI) is more common in diabetes and about 25% of diabetic women have asymptomatic bacteruria (four times the frequency in non-diabetic women). Asymptomatic bacteruria was observed among many cases in our study also. Escherichia coli is the most common pathogen. UTI in diabetes may be asymptomatic, or present with dysuria, increased frequency or urgency (lower UTI) or flank pain, fever and vomiting (upper UTI).

Respiratory tract infections may not be more frequent in diabetes, but bacteraemia, delayed resolution and recurrence are frequent.

Common infections with increased incidence in Diabetic patients:

a) Urinary tract infections
b) Respiratory tract infections
c) Soft tissue infections

Infections predominantly occurring in diabetic patients:

a) Malignant otitis externa
b) Rhinocerebral mucormycosis
c) Necrotizing fasciitis
d) Fournier's gangrene
e) Emphysematous cholecystitis
f) Emphysematous pyelonephritis; pyelitis and cystitis
g) Infections in the diabetic foot.

Predisposing Factors For Infections In Diabetes Mellius

Primary factors:

- Defects in the following functions of neutrophils
  - Adherence
  - Chemotaxis
  - Phagocytosis
  - Bactericidal activity
- Myeloperoxidase deficiency

- Complement pathway defects
- Cytokine-mediated(eg. Interleukin-1, tumor necrosis factor)

Secondary factors:

- Ketoacidosis
- Intravascular access lines
- Antibiotic misuse/resistance
- Frequent hospitalization
- Peripheral vascular disease
- Neuropathy
- Gastroparesis and aspiration
- Indwelling urinary catheters
- Chronic renal failure and dialysis
- Total parenteral nutrition

Neutrophil Dysfunctions In Diabetes Mellitus

i) *Adherence*:

Peterson *et al,*[23] observed that the neutrophils of patients with poorly controlled diabetes exhibited impaired adherence to a glass-wool column, and Bagdade[24] showed an enhancement of adherence of neutrophils to a nylon-fiber column following an improvement in the control of blood glucose levels. Andersen *et al*[25] showed similar results on examining the ability of neutrophils to bind to bovine aortic endothelium.

ii) *Mobilization and Chemotaxis*:

There is reduced mobilization and chemotaxis of neutrophils in diabetes patients as shown by several investigators.

Using the Rebuck skin window technique[26](an abrasion created on the volar forearm, and sterile coverslips being serially applied over several hours, and the coverslip smears stained) or its modification,[1] researchers studied the

mobilization of neutrophils to the area of inflammation by microscopic examination of the stained smears over time. They found reduced mobilization in diabetes patients irrespective of whether diabetes was well controlled, poorly controlled or complicated by ketoacidosis.

A modified tissue-culture chamber method,[27] where a chemotactic index was derived by comparing the original number of neutrophils with the number that had completely crossed a filter barrier in response to chemoattractants, also showed that neutrophils of patients with diabetes had a lower chemotactic index (i.e., diminished response), without any correlation with fasting blood glucose levels or type of therapy. Incubation of neutrophils of diabetics with insulin improved the chemotactic index.[28]

Other techniques to study chemotaxis like subagarose technique[29,30] also showed depressed chemotactic index in diabetics, which was correctable with intensive treatment of diabetes.

iii) *Phagocytosis*:

Decreased phagocytosis, a feature of diabetes, was noted when washed neutrophils of poorly controlled diabetics and an equal number of Streptococcus pneumonia were incubated in 90% serum (phagocytosis being considered to be present if at least one bacterium was ingested). Incubation of neutrophils of diabetics with serum of healthy controls showed normal phagocytosis and incubation of control neutrophils in serum of diabetics chowed reduced phagocytosis. This suggested the possibility of an

opsonization defect affecting the neutrophils of diabetics as supported later by the works of Rayfield *et al*[31] and Davidson *et al*[32].

Using the lysostaphin assay technique, which allows differentiation between phagocytosis and intra-cellular killing, Tan *et al*[33] demonstrated a defect in phagocytosis of Staphylococcus aureus in diabetics, which showed no correlation with the level of glycemic control or history of recurrent infections.

Alexiewicz *et al*[34] demonstrated an inverse relationship between phagocytic activity and fasting glucose levels, as well as a reduced phagocytic function.

Defects in adherence and phagocytosis did not correlate with levels of glycosylated hemoglobin (HbA_{1c}).[25,32]

iv) *Bactericidal activity*:

Early studies such as that of Dziatkowiak *et al*,[35] compared the number of live Staphylococcus aureus in a granulocyte with the total number engulfed , to calculate the proportion of organisms killed.

Studies using single low ratio of bacteria to neutrophils, demonstrated diminished killing by the neutrophils of the patients with diabetes while others did not.[28] Repine *et al*,[36] used five different ratios of bacteria to neutrophils. Study patients included infected and non-infected individuals with and without diabetes. Cells were incubated with Staphylococcus aureus for one hour after which colonies were counted. The rates of intracellular killing of bacteria by the neutrophils from uninfected controls and by the neutrophils of persons with

well controlled diabetes were comparable. Neutrophils from uninfected patients with poorly controlled diabetes functioned less well, especially when the higher ratios of bacteria to the neutrophils were used. Although the bactericidal activity of neutrophils from infected patients with well controlled diabetes was similar to that of uninfected controls, they did not display the increase in killing activity seen in neutrophils of infected patients without diabetes. The bactericidal function of neutrophils from infected patients with poorly controlled diabetes was the lowest of all groups.

Production of superoxide anions and other oxygen derived free radicals during respiratory burst by stimulated neutrophils produce chemiluminiscence, which was studied by Shah *et al*[37] using neutrophils from patients with diabetes and controls. In resting neutrophils the chemiluminescence of cells was comparable in both groups. But on stimulation, the neutrophils from patients with diabetes showed a blunted response with regard to superoxide production and chemiluminescence. The control of diabetes improved chemiluminescence[38] and also other neutrophil functions.[36, 39] Cross incubation serum studies effected no change, suggesting that an intracellular defect rather than an inhibitory serum factor might have been present. Whether this bactericidal defect in diabetes is a predisposing factor for infections is uncertain, as the defect is seen in diabetes, irrespective of whether they have had recurrent/serious infections.[40]

Li.Y.M.[41] suggested that advanced glycation end products (AGEs) associated with diabetes may bind to specific motifs common to lactoferrin,

lysozyme and other antimicrobial proteins found in neutrophils and interfere with antimicrobial function of these host defence molecules.

v) Other mechanisms for neutrophil defects in diabetes:

These include a state of persistent low level activation of the neutrophils by AGEs. This is evidenced by an increased concentration of neutrophil elastase, increased activity of neutrophil alkaline phosphatase and an increased rate of neutrophil oxygen consumption among unstimulated neutrophils of patients with diabetes. This hyperexcited state leads to spontaneous activation of the respiratory burst and release of neutrophil granule components that can be detrimental in two ways:

a) It may lead to 'burnt out' of neutrophils that respond less vigorously when stimulated by an infectious pathogen.

b) It may initiate pathological processes leading to vascular injury[42]

NBT test in diabetes mellitus:

Relatively a few authors have done works using NBT test in diabetes mellitus.

Kruszewski *et al*[43] in 1979 studied 44 diabetic patients and found normal range of NBT scores in those without infection and significantly higher values in those with bacterial infection.

Lechowski *et al*[44] in 1991 observed that the NBT reducing value of circulating phagocytes in diabetic dogs was increased (P less than 0.05),

whereas in these cells increase in NBT reduction following stimulation was decreased (P less than 0.05). It is suggested that in diabetic dogs changes in activity of circulating and tissue phagocytes may occur.

Nurun Nabi *et al*[45] in 2005 studied neutrophil dysfunction in streptozocin induced type1 diabetic rats and found that diabetic rats showed higher NBT reduction than controls which correlated with polarization assay in which the neutrophils of diabetic rats were significantly more polarized at baseline level compared to control rats.

Larijani *et al*[46] in 2007 compared the effect of NBT between type2 diabetes patients and healthy controls. They found that NBT test was significantly high in diabetics compared to controls. But stimulation(by endotoxin) resulted in inadequate rise of NBT score compared to controls.

Other applications of the NBT test:

NBT test is an useful indicator of infection in patients with underlying *cancer.* This was shown by Lehane *et al*[47] who observed that cancer patients with infection showed a significantly higher score than those without infection. Similar results were demonstrated by Jadrezejczak *et al*.[48]

Jedrzejczak *et al*[49] compared the NBT scores between untreated cancer patients with or without bacterial infection and those on radiotherapy and/or *chemotherapy*. NBT scores were raised in cancer patients with bacterial infection irrespective of treatment. Thus the NBT test was an useful tool for infection screening in cancer patients. However the test could not be done in severe neutropenia

Patients with sickle cell anemia have an increased susceptibility to bacterial infections. There are differing opinions by different researchers. Walter et al[50] found a significant difference in the mean NBT values between sickle cell anemia patients with bacterial infection and those without infection, whereas Wajima T[51] observed low NBT scores in sickle cell patients in painful crisis with bacterial infection. Akinyanju[52] had similar results as that of Walter et al except that he found low scores in painful crisis (similar to Wajima) and early osteomyelitis in sickle patients.

Wantzin[53] observed a negative effect of prednisolone on the NBT response as seen in both non-stimulated and stimulated NBT tests despite the fact that a pronounced neutrophil granulocytosis occurred on stimulation with toxins in the stimulated test groups. The percentage of NBT stained neutrophils and absolute count of NBT stained neutrophils were reduced.

Hypertriglyceridemia depresses the granulocytic activity as was demonstrated by Broden *et al*.[54] He observed that significant number of patients with acute bacterial pancreatitis, despite having fever, granulocytosis and elevated ESR had a lower than normal NBT scores.

Normal pregnant women have NBT scores similar to that of healthy volunteers.[10]

Comparison of NBT scores with total leucocyte count, differential leucocyte count, ESR and neutrophil alkaline phosphatase (NAP) scores was conducted by Douwes *et al*.[55] In bacterial infection, the NBT scores were

significantly raised, whereas such a rise was not observed with the TLC, ESR or LAP scores.

Kucharska *et al*[56] studied NBT test in bacterial and viral meningitis. They found a significantly higher NBT reduction in neutrophils of bacterial compared to viral meningitis, whereas NBT scores in monocytes/macrophages were raised in both types of meningitis.

Other factors which affect the NBT scores:

Earlier, when heparin was used as the anticoagulant, it was observed that higher concentrations of it resulted in falsely high NBT scores [57,58]. Also, experiments comparing EDTA and heparin have shown that while formazan staining was satisfactory with the latter, cell definition was relatively poor with cell destruction and clumping rendering counting difficult. Extravasation of formazan from neutrophils has been seen in EDTA anticoagulated blood. The use of Ficoll sucrose polymer in the NBT reagent was found to prevent this leak by effecting membrane stabilization[6].

Time lag between blood collection and the performance of the test resulted in falsely higher NBT scores[57]. The mean NBT scores increased when blood samples were kept at room temperature for more than 2 hours, but remained virtually constant until 12 hours when stored at 4°C. After 12 hours the mean NBT scores fell slightly at both temperatures.[60]

The effects of *pH*, composition of the buffer used, and NBT dye concentration have also been investigated. Phosphate-buffer with a pH of 7.2 containing 0-1% NBT dye gave the most reliable results [57,59,60]. Both resting

and stimulated neutrophils showed an increase in NBT positivity with higher concentrations of NBT dye.[6]

NBT scores increase with increasing incubation time. In resting neutrophils a 20 min incubation at 37°C resulted in a mean NBT score of 10% whereas a period of 30 min gave a score of 20%.[60] As cell destruction and clumping enhanced with higher incubation times, 30 min incubation at 37□C followed by 15 min at room temperature has been considered as optimal.[61]

Presence of specific antibodies and complements in serum have been shown to raise the NBT scores[62].

Differences in *counting* techniques of NBT positive neutrophils has been an important reason for variations in results between workers.[59]

In view of the fact that various factors and conditions alter the NBT test results, the scores should be interpreted with caution and in the context of other laboratory test results and clinical picture of the patient.[63]

Various methods/modifications of performing the NBT test:

a) The spontaneous NBT *test* is one were the neutrophils' response to the addition of NBT dye is studied without the addition of any external stimulants. Instead of whole blood, the use of buffy coat preparation has the advantage of performing the test even in the presence of neutropenia and does not impair the cytological quality of preparation.[6]

b) The stimulated NBT test is one were the neutrophils from the same subjects are stimulated in vitro by adding endotoxin, and the rise in

percentage of cells reducing NBT determined.[64] In certain situations (usually when a very low or completely negative result is obtained by the spontaneous test), it is necessary to perform the stimulated test in order to differentiate a transient lack of responsiveness from a congenital defect. A good example of the former might be a patient on high dose of corticosteroids and of the latter the classic example is chronic granulomatous disease.[65,66]

c) The cytocentrifuge NBT test is a modification of the technique were the anticoagulated whole blood is dissolved with the NBT reagent and hemolysed. The hemolysate is then cytocentrifuged and smears are spun on to glass slides. This method reduces the risk of non-specific stimulation of neutrophils and also bypasses cell membrane disruption which could occur when subjected to the lateral shear involved in manual smearing.[60]

MATERIALS AND METHODS

This prospective study was conducted in the Goschen Institute of Pathology, Madras Medical College, from 2006-2008. The study included 127 subjects divided into 4 categories. 31 were non-diabetics without bacterial infection (c), 31 were non-diabetic patients with a clinical diagnosis of bacterial infection (i), 33 patients had diabetes (d) with no bacterial infection (asymptomatic infections like UTI excluded by urine culture).and 32 patients had diabetes and bacterial infection (di). An attempt had been made to select patients with similar infections in non diabetics (i) and diabetics (di).

Inclusion Criteria: The bacterial infection was confirmed by bacteriological isolation through culture or other relevant tests. The selected diabetic study population had diabetes for at least 2 years duration, with or without treatment.

Exclusion Criteria: The following were excluded from the study population

1) Patients with subclinical infection

2) Patients who had completed a full course of antibiotics

3) Patients in convalescence

4) Duration of diabetes of <2 years.

The study population were subjected to the lab investigations which included total leucocyte count(normal range of total leucocyte count : 4.0-10.0x 10^9 /L), differential leucocyte count(normal: neutrophils40-80%; lymphocytes:20-40%; monocytes:2-10%; eosinophils:1-6%; and basophils1-

2%), erythrocyte sedimentation rate (normal range for males:1-25mm/hr; for females:0-17mm/hr), fasting blood sugar (normal range :75-115mg/dl; fasting levels in diabetes mellitus: >125mg/dl)[67] and bacteriological tests and NBT test. Absolute neutrophil count (ANC) was derived from TLC and DLC (normal ANC= 2.0-7.0x 10^9/L i.e.,40%-80% of total leucocyte count)[68-70].

NBT Test Procedure

Reagents :

- Solution A: 0.2 M Monobasic sodium phosphate (31.2g aH2PO4.2H2O/Litre)
- Solution B: 0.2 M Dibasic sodium phosphate (28.3g Na2HPO4/Litre)
- Deionized water
- Nitroblue tetrazolium dye (crystalline grade III, Sigma chemical company)
- Aqueous safranin 0.5%

Preparation of NBT Solution:

To 20ml of solution A, 80ml of solution B was added. This solution was dissolved in 100ml deionized water, forming the working buffer with a final pH of 7.2 . NBT dye was dissolved at a concentration of 0.2% to the working buffer, and stored at 4^0C.

The method followed in this study was a modification of that of Gordon et al.[6] 4ml venous blood was collected in EDTA (2mg/ml of blood) bottles, from each subject and transferred to plastic tubes, centrifuged at 3000rpm for 8 minutes. The separated buffy coat was transferred using plastic pipettes into separate plastic tubes. Equal amount of NBT solution was added , mixed gently and incubated at 37^0C for 30 minutes and left at room temperature for 15 minutes. The mixture was mixed again gently, a drop of which was smeared on a glass slide, dried, heat fixed and counterstained with safranin for 2 minutes that stains the nucleus of leucocytes red. The smears were them washed and left to dry.

Counting techniques:

The slides were examined under oil immersion objective (1000x) and 100 neutrophils (identified by the number of nuclear lobes) were observed sequentially.

The NBT scoring was done by two scoring systems:

(A) 1^{st} (Classic) scoring system

(B) 2^{nd} (Grading) system

(i) In the first scoring system (classic system), the cells exhibiting discrete fine/coarse particulate cytoplasmic distribution of formazan as blue/black granules, in addition to dense deposits of formazan[6] were counted as NBT positive cells. Those cells which showed no formazan deposits were counted as NBT negative.The number or nature of formazan granules was not taken into consideration. The NBT score was

expressed in percentage. Only neutrophils with intact cell membranes were taken for observation.

NBT score in healthy volunteers (range) in our study: 6-25%

(ii) In the second scoring system (grading system) Grading of the number and nature of formazan granules in NBT stained neutrophils was attempted similar to that of NAP (Neutrophil alkaline phosphatase) scoring.[71] The intensity of the reaction in neutrophils varies from negative to strongly positive with fine/coarse granules filling the cytoplasm and overlying the nucleus. An overall score is obtained by assessing the stain intensity in 100 consecutive neutrophils, with each neutrophil scored on a scale of 0-4 as follows:

0 - Negative, no granules

1 - Occasional granules scattered in the cytoplasm

2 - Moderate number of granules <25% of cytoplasm

3 - Numerous granules occupying < 50% cytoplasm

4 - Heavy positivity with numerous coarse granules crowding the cytoplasm, frequently overlying the nucleus > 50% of cytoplasm

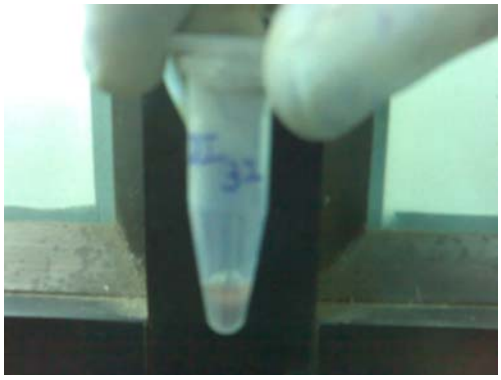
The overall possible score will range between 0 and 400 per 100 cells.

The NBT grading score in healthy volunteers in our study : 12-167.

The NBT scores and the Absolute neutrophil counts of the 4 study groups were compared and results tabulated and statistical analysis done using the Z test.

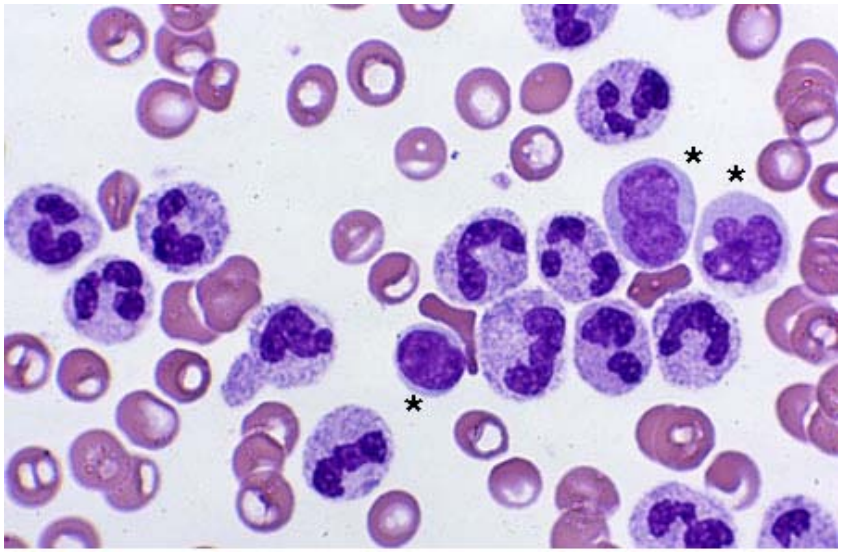
NBT dye with buffer solution

Buffy coat after centrifuging

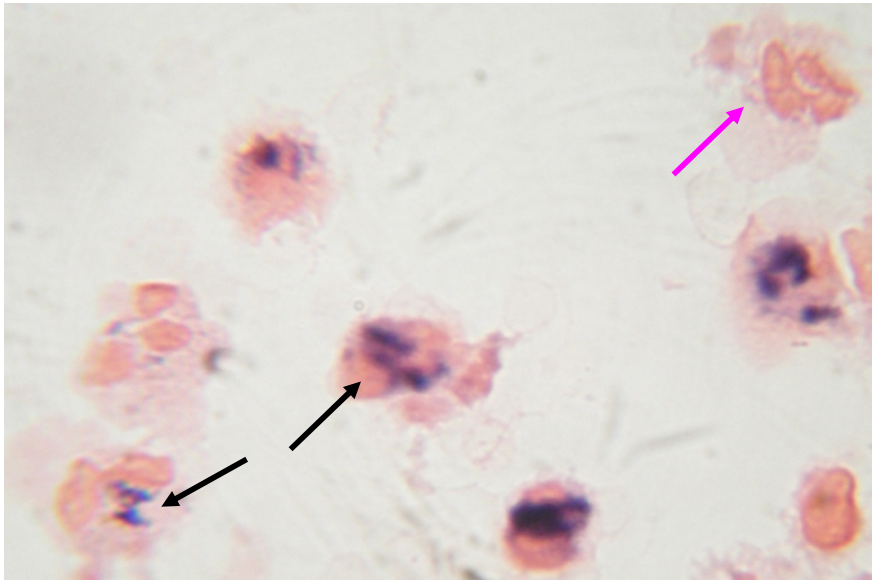


Buffy coat with NBT –buffer mixture prior to incubation

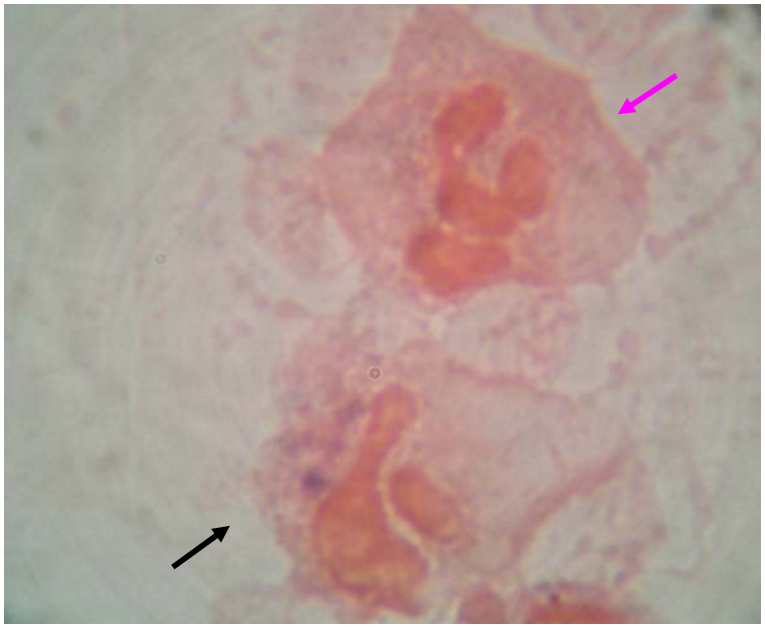
Buffy coat with NBT-buffer mixture after incubation, turning blue



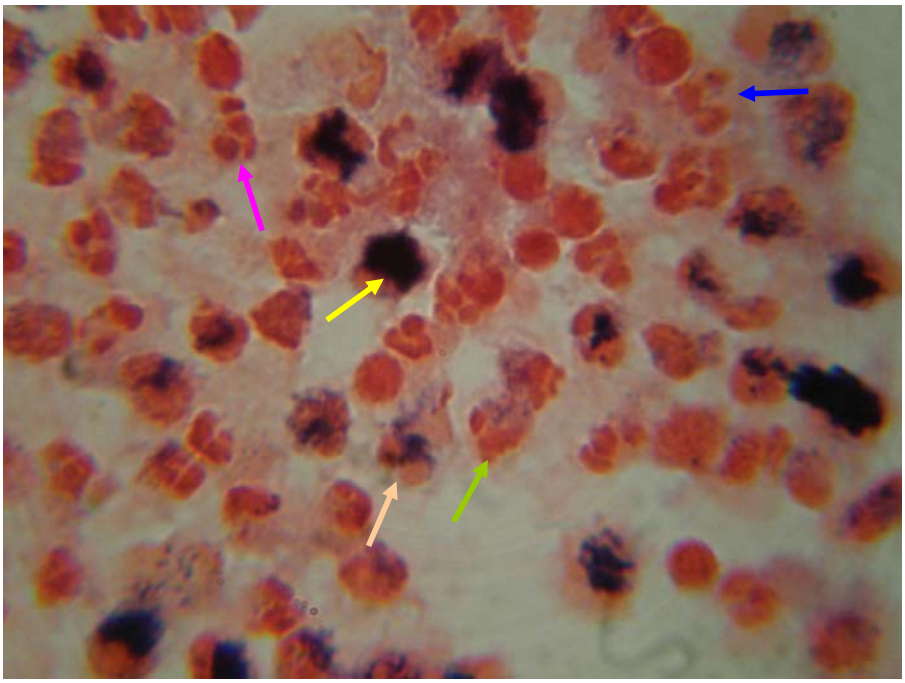
Peripheral smear showing neutrophilia, with neutrophils exhibiting toxic granules(1000x)



Peripheral blood buffy coat smear, NBT stain (1000x) showing NBT (Blue black granules / deposits) positive neutrophils (↗) and a NBT negative neutrophil (↗)



Peripheral blood buffy coat smear, NBT stain (1000x) showing a positively stained neutrophil (1 grade) ( ) and a NBT negative neutrophil ( )



Peripheral blood buffy coat smear, NBT stain (1000x) showing several NBT stained neutrophils (0 grade), (1+ grade) (2+ grade) (3+ grade) and (4+ grade)

RESULTS

A. Results of NBT scoring based on the 1st (Classic) Scoring System

Table 1 : Experimental categories

Category	Nomenclature	n
Non-diabetics without bacterial infection	c	31
Non-diabetics with bacterial infection	i	31
Diabetics without bacterial infection	d	33
Diabetics with bacterial infection	di	32

Non-diabetics without infection (C):

This group (c) of 31 subjects in our study, consisted of 17 males and 14 females. The age range of this group was 18 to 41 years, with a median of 20 years. All were healthy with no signs and symptoms of diabetes or infection.

The mean NBT score for this group was 21.8% (confidence interval of 21.8+/-5).

There was no significant difference ($P>0.05$) in the mean NBT scores between the males (20.6%) and females (23.3%).

The mean absolute neutrophil count (ANC) was $4.3x10^9$/L.

The results are summarised in Table 2.

Table 2

n	Mean NBT %	NBT Confidence Interval (Mean+/-2SE))	Mean ANC(x10^9/L.)	ANC Confidence Interval (Mean+/-2SE)
31	21.8	21.8+/-5	4.3	4.3+/-0.676

Non-Diabetics with Infection (i) :

31 patients in our study had a clinically and microbiologically proved bacterial infection of which 24 were males and 7 females with their age ranging from 19 to 75 years. The distribution of cases are tabulated below.

Table 3

Diagnosis	n
Wound infection	11
Abscess	3
Respiratory infection	3
Ulcer foot	3
Urinary tract infection	2
Leptospirosis	2
Enteric fever	2
Fournier's gangrene	1
Necrotizing fasciitis	1
Meningoencephalitis	1
Septicemia	1
Vaginitis	1
TOTAL	31

The mean NBT score in this category (i) was 76.5% with a confidence interval of 76.5+/-6.4.

The mean NBT score in males was 76.9% and females was 75.1% with no significant difference(P>0.05).

The mean ANC was $6.1x10^9$/L.The results of summarised in Table 4.

Table 4

n	Mean NBT %	NBT Confidence Interval (Mean+/-2SE))	Mean ANC ($x10^9$/L.)	ANC Confidence Interval (Mean+/-2SE)
31	76.5	76.5+/-6.4	6.1	6.1+/-1.61

Diabetes without Infection (d):

33 diabetic patients without infection were selected of whom 16 were males and 17 were females in the age range of 27 to 78 years.

The mean NBT score in this group was 32.7% with 95% of the scores falling between 25.3% and 40.1% .

The mean NBT score of the males was 26.06% and females was 39% with no significant difference (P>0.05) between them.

The mean ANC was $4.6x10^9$/L. The results are summarised in Table 5.

24 cases had FBS<200mg/dl whereas the rest had higher fasting sugar levels.

Table 5

n	Mean NBT %	NBT Confidence Interval (Mean+/-2SE))	Mean ANC ($x10^9$/L.)	ANC Confidence Interval (Mean+/-2SE)
33	32.7	32.7+/-7.4	4.6	4.6+/-0.528

Diabetes with Infection (di):

This category of 32 patients had diabetes and bacterial infection of which 19 were males and 17 females, with age ranging from 20 to 85 years. The distribution of cases are listed in Table 6.

Table 6

Diagnosis	Number of cases
Diabetic foot	16
Urinary tract infection	11
Wound infection	1
Melioidosis	1
Peritonitis	1
Enteric fever	1
Osteomyelitis	1
TOTAL	32

The mean NBT score in (di) group was 63.4% with 95% of the scores between 55.6% and 71.2%

The mean NBT of the males was 59% and not significantly lower than that of females (mean NBT=69.8%).

The mean ANC was $7.6x10^9$/L

The results are summarised Table 7.

Table7

n	Mean NBT %	NBT Confidence Interval (Mean+/-2SE))	Mean ANC ($x10^9$/L.)	ANC Confidence Interval (Mean+/-2SE)
32	63.4%	63.4+/-7.8	7.6	7.6+/-1.676

Table 8

Case (n) distribution in 1st (classic) scoring system

NBT score range	c	i	d	di
0-20	19	0	8	1
21-40	9	2	18	6
41-60	2	4	3	8
61-80	1	11	2	6
81-100	0	14	2	11
Total (n)	31	31	33	32

In this classic system, as expected, more number of non-diabetics without infection (19 cases) had their scores less than 20, the diabetics without infection (18 cases) between 21-40, the diabetics with infection (20 cases) between 21-80, while most non-diabetics with infection (25 cases) had scores between 61-100.

Comparison of Results of the 4 Groups

Comparison of the results of non-diabetics without (c) and with infection(i) :

Table 9

Comparison of NBT scores

	Mean NBT (%)	**σ**	**Standard error of difference between 2 means**	**Z score**	**P value**
Non-diabetics without infection (c)	21.8	14	4.0	13.495	<0.05
Non-diabetics with Infection(i)	76.5	17.7			

Table 10:

Comparison of ANCs

	Mean ANC	**σ**	**Standard error of difference between 2 means**	**Z score**	**P value**
Non-diabetics without infection (c)	4.3 x10^9/L	1.9	878	1.992	<0.05
Non-diabetics with Infection(i)	6.1 x10^9/L	4.5			

The mean NBT scores of non-diabetics with infection (i) was 76.5% and was found to be significantly higher than that of non-diabetics without infection (21.8%). As expected, the neutrophil count was higher among those with bacterial infection than controls.

Figure 1 : Comparison of NBT scores in non-diabetics without (c) and with infection (i)

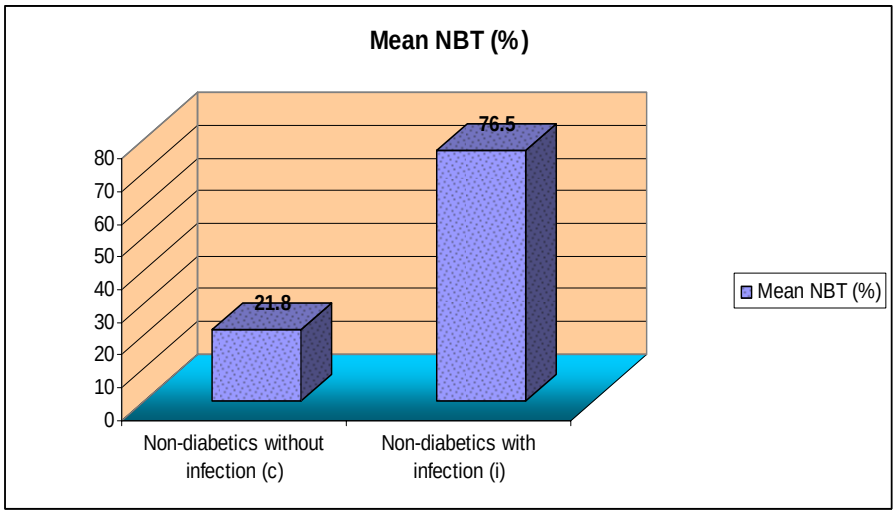


Figure 2 Comparison of ANC in non-diabetics without (c) and with infection (i)

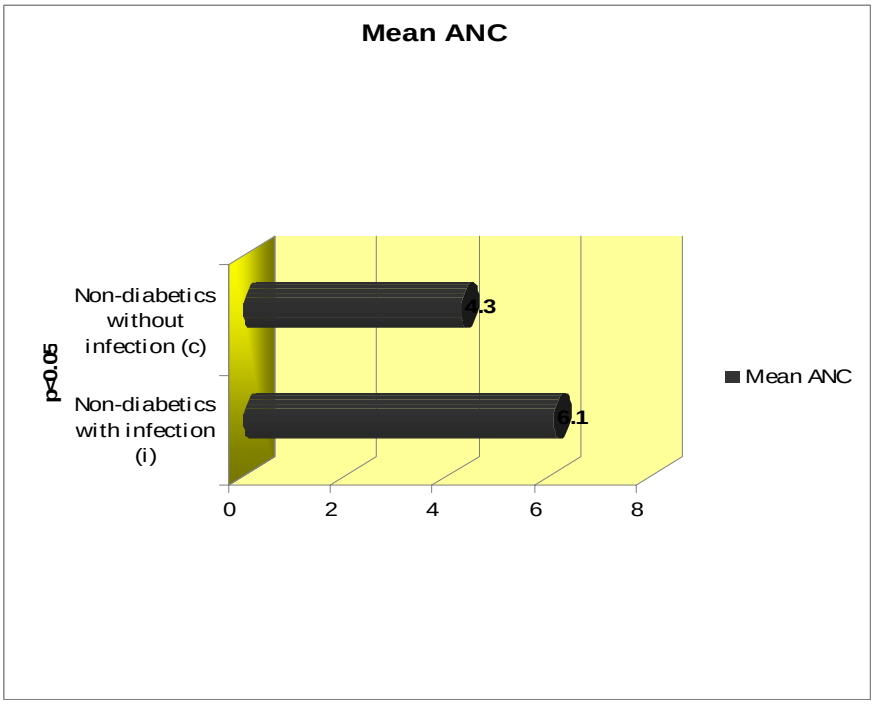
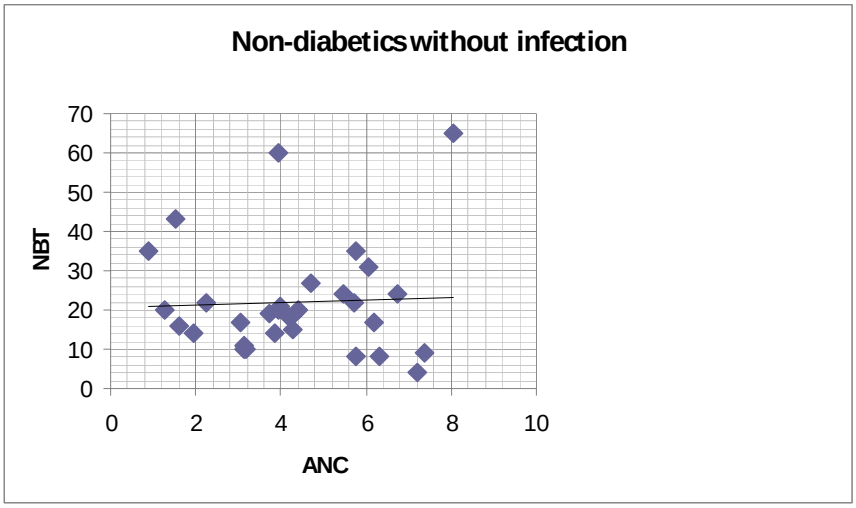
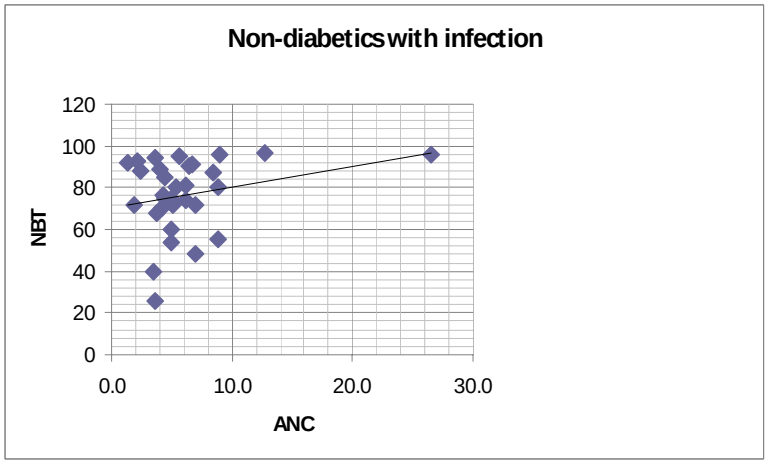


Figure 3 Scatter diagram comparing NBT and ANC in non-diabetics without infection



From the scatter diagram above it could be seen that with an increase in ANC there was no significant rise in NBT scores.

Figure 4 Scatter diagram comparing NBT and ANC in non-diabetics with infection



From the diagram above, as was expected, in the presence of infection there was an increase in NBT score with an increase in ANC - reflection of a normal neutrophil functional response.

The Comparison of diabetics without (d) and with infection (di):

Table 11:

Comparison of NBT scores

	Mean NBT (%)	**σ**	**Standard error of difference between 2 means**	**Z score**	**P value**
Diabetics without infection (d)	32.7	20.9	5.3	5.792	<0.05
Diabetics with infection (di)	63.4	21.8			

Table 12

Comparison of ANCs

	Mean ANC	**σ**	**Standard error of difference between 2 means**	**Z score**	**P value**
Diabetics without Infection(d)	4.6 $x10^9$/L	1.5	870	3.486	**<0.05**
Diabetics with infection(di)	7.6 $x10^9$/L	4.7			

As shown in above the mean NBT scores and ANC of diabetics with infection (di) was significantly higher than diabetics without infection(d).

Figure 5 : Comparison of NBT scores in diabetics without (d) and with (di) infection

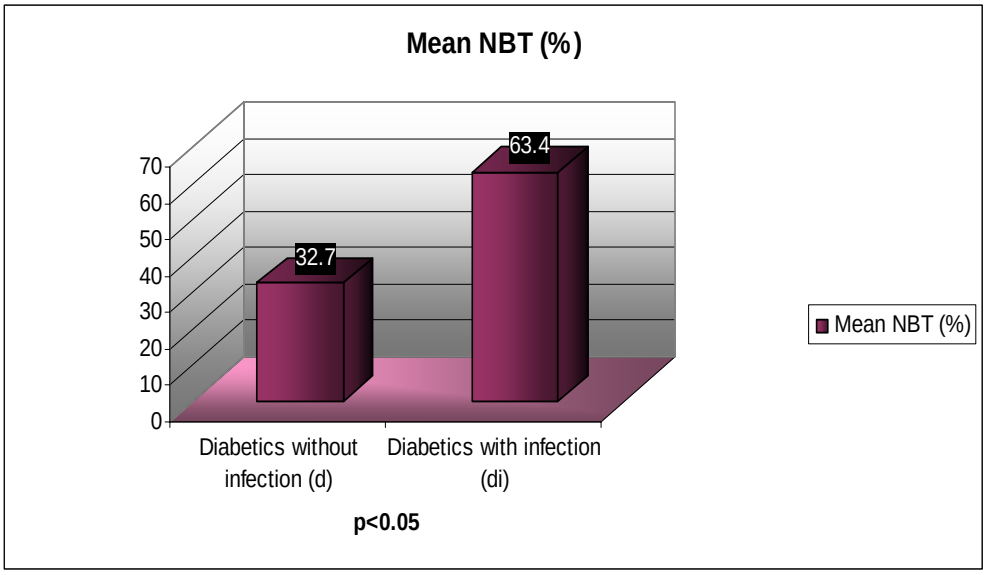


Figure 6 : Comparison of ANCs in diabetics without (d) and with (di) infections

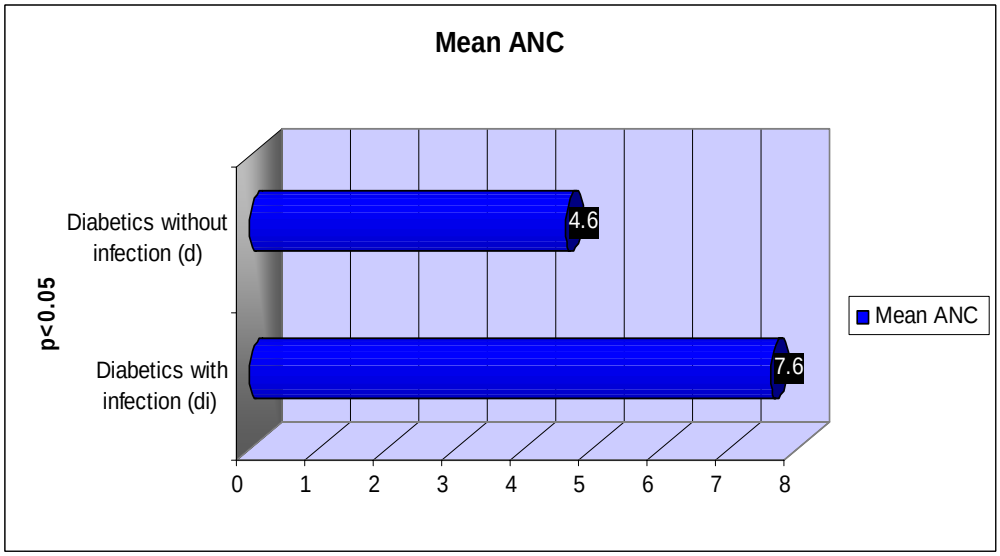
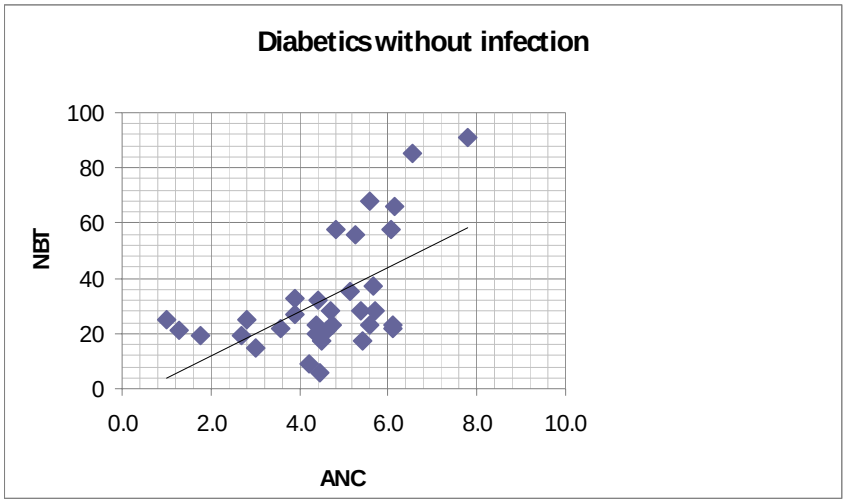
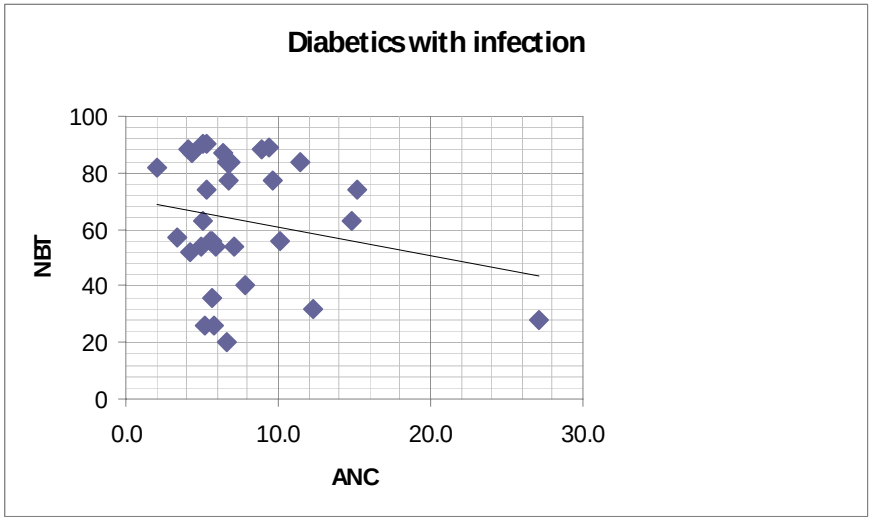


Figure 7. Scatter diagram comparing NBT and ANC in diabetics without infection



From this diagram it was observed that with an increase in ANC, there was an increased NBT score.

Figure 8. Scatter diagram comparing NBT and ANC in diabetics with infection



From this table it is observed that with increasing neutrophil counts there is a decrease in NBT scores, probably a reflection of premature exhaustion and consequent impaired neutrophil function in diabetic patients with infection.

Comparing of non-diabetics (c) and diabetic(d) without infection :

Table 13 Comparing the NBT scores

	Mean NBT (%)	**σ**	**Standard error of difference between 2 means**	**Z score**	**P value**
Non-diabetics without infection (c)	21.8	14	4.4	2.465	<0.05
Diabetics without infection (d)	32.7	20.9			

As shown above the mean NBT scores of diabetes without infection(d) was 32.7% and was significantly higher (P<0.05) than that of non-diabetics without infection(21.8%) .

Table 14 Comparing of ANCs

	Mean ANC	**σ**	**Standard error of difference between 2 means**	**Z score**	**P value**
Non-diabetics without infection (c)	4.3 $x10^9$/L	1.9	430	0.637	>0.05
Diabetics without infection (d)	4.6 $x10^9$/L	1.5			

However no significant difference was found between their mean ANCs.

Figure 9 : Comparison of NBT scores in nondiabetics (c) and diabetics (d) without infection

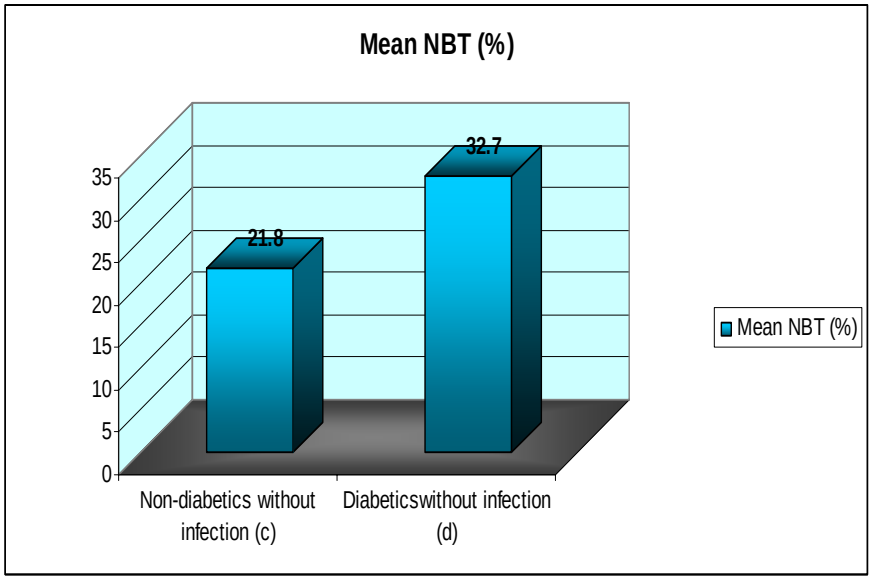
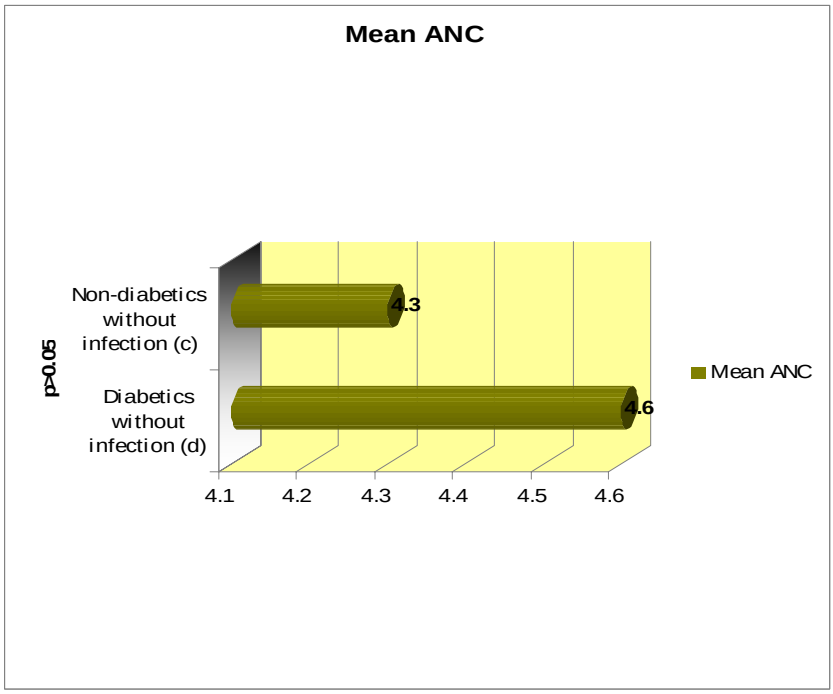


Figure 10 : Comparison of ANCs scores in nondiabetics (c) and diabetics (d) without infection



Comparison of non-diabetics (i) and diabetics(di) with infection :

Table 15 Comparison of NBT scores

	Mean NBT (%)	**σ**	**Standard error of difference between 2 means**	**Z score**	**P value**
Non-diabetics with infection(i)	76.5	17.7	4.9	2.622	<0.05
Diabetics with infection(di)	63.4	21.8			

The mean NBT in diabetics with infection is significantly lower than that of non-diabetics with infection ($P<0.05$).

Table 16 Comparison of ANC

	Mean ANC	**σ**	**Standard error of difference between 2 means**	**Z score**	**P value**
Non-diabetics with infection(i)	6.1 $x10^9$/L	4.5	1160	1.342	>0.05
Diabetics with infection(di)	7.6 $x10^9$/L	4.7			

From the above two tables, it is seen that though the diabetics with infection (di) have a lower NBT score compared to non-diabetics with infection (i), they have a slightly higher (though not significant) mean ANC compared to the latter. This higher mean ANC in diabetics with infection (di) is probably a reflection of a compensatory increase to a compromised neutrophil function .

Figure 11 : Comparison of NBT scores in non-diabetics (i) and diabetics (di) with infection

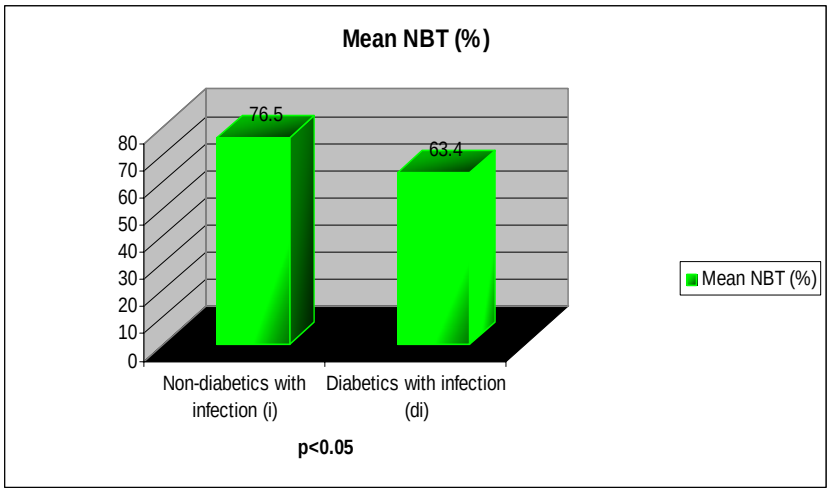
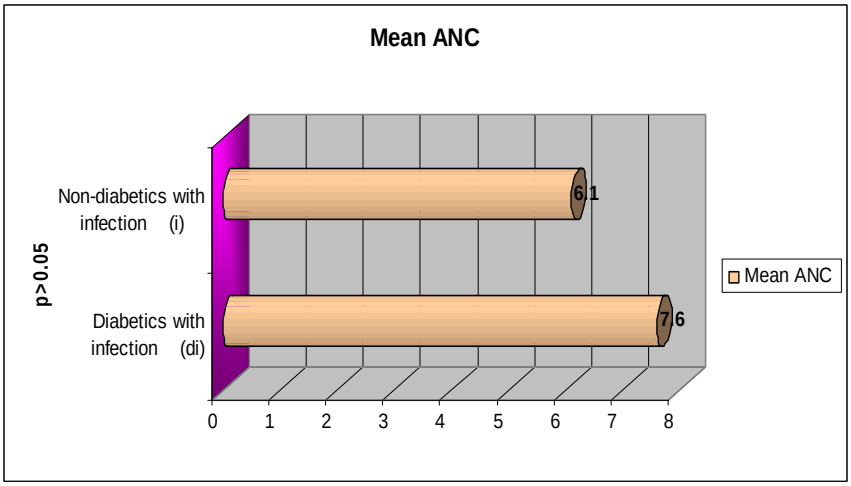


Figure 12 : Comparison of ANCs in non-diabetics (i) and diabetics (di) with infection



B. Results of NBT scoring based on the 2nd (grading) scoring system:

The range and mean values for each of the four categories were: non-diabetics without infection 12-167 (mean 82); non-diabetics with infection 48-348 (mean 228.5); diabetics without infection 51-260 (mean 117.2); diabetics with infection 106-311(mean 198.8) which are given in Table 17.

Table 17 Mean NBT scores based on the grading (2nd scoring) system

	Non-diabetics without infection (c)	**Non-diabetics with infection (i)**	**Diabetics without infection (d)**	**Diabetics with infection (di)**
Mean score	82	228.5	117.2	198.8

From the above table it was observed that the mean NBT scores of non-diabetics with infection (i) was higher ($P<0.05$) than that of non-diabetics without infection (c). the mean NBT score of diabetics was higher ($P<0.05$) in those with infection (di) compared to those without infection (d). In those without infection, the mean NBT score was higher ($P<0.05$) in diabetics (d) compared to non-diabetics (c). And finally, among those with infection the score was higher in non-diabetics (i) compared to diabetics (di).

Table 18

NBT Score range	**Case (n) distribution in NBT Grading system**			
	C	**i**	**d**	**di**
0-50	11	1	0	0
51-100	7	1	15	0
101-150	7	2	11	10
151-200	3	5	4	7
201-250	0	9	2	7
251-300	0	8	1	5
301-350	0	4	0	2
351-400	0	0	0	0
Total (n)	28	30	33	31

In this grading system also, the highest score ranges in the various categories were: non-diabetics without infection (11 cases) 0- 50, the diabetics without infection (15 cases) between 51-100, the diabetics with infection (10 cases) between 101-150 while most non-diabetics with infection (17 cases) had scores between 200-300. Hence, we observe in this scoring system (similar to the first), that though higher scores are obtained in most cases of infection, cases of diabetes with infection had relatively lesser scores compared with non-diabetics with infection.

DISCUSSION

Till date the utility of NBT test had been in the diagnosis of chronic granulomatous disease (CGD). Being technically simple and fairly economical, an attempt has been made to extrapolate this test's usefulness in detecting and quantifying neutrophil function in patients with diabetes mellitus.

Comparison with standard studies between non-diabetics with (i) and without (c) infection

Table 19 Comparison of mean NBT scores (classic scoring system).

Study	Mean NBT% (i)	Mean NBT% (c)	P value
Gordon *et al*[6] 1973	34.2	6.1	<0.05
Gordon *et al*[72] 1974	21	5	<0.05
Gordon *et al*[60] 1975	60	18	<0.05
Trojan *et al*[13] 1975	72	33	<0.05
Bjorksten *et al*[73]1975	19	13	<0.05
Hellum KB[20] 1977	29.8	5.3	<0.05
Yun Woong Ko *et al*[74] 1977	10.6	3.2	<0.05
Akinyanju *et al*[52] 1985	41	20	<0.05
Present study	76.5	21.8	<0.05

Table 20 Comparison of mean ANC

Study	Mean ANC (i)	Mean ANC (c)
Yun Woong Ko *et al* 1977	10.4 $x10^9$/L	4 $x10^9$/L
Present study	6.1 $x10^9$/L	4.3 $x10^9$/L

The significant difference in the mean NBT scores between non-diabetics with infection (76.5%) and non-diabetics without infection (21.8%), obtained in our study was similar to that of various standard studies as shown in the table 19. But some studies showed lower NBT scores which might be due to differences in procedure and sample size. With reference to counting techniques, some studies[6] considered only those cells exhibiting a discrete particulate cytoplasmic distribution of formazan along with those showing dense deposits of formazan (block positivity) as positive cells whereas in our study both fine & coarse granules and dense deposits were taken as positive.

This rise in NBT scores seen in the presence of infection in our study as with others, was in accordance to the fact that neutrophils, on stimulation by an infectious pathogen, have an increased bactericidal activity.[7] Similarly as shown in table 20, a significant difference in mean ANCs between nondiabetics without and with infection was found in the standard study as in ours.

Comparison with a standard study between diabetics without (d) and with (di) infection

Table 21 Comparison of NBT scores and ANC

		Yun Woong Ko *et al* 1977	**Present study**
Diabetics without infection (d)	No. of cases	10	33
	Mean NBT %	2.6	32.7
	Mean ANC $x10^9$/L	3.5	4.6
Diabetics with infection (di)	No. of cases	10	32
	Mean NBT%	4.6	63.4
	Mean ANC $x10^9$/L	9.9	7.6

As shown in the table above the mean NBT scores and ANC of diabetics with infection(di) was significantly higher than diabetics without infection(d) in our study. In the study by Yun Woong Ko *et al*[74], although the mean NBT was higher in diabetics with infection it was not statistically significant probably due to a small sample size . The diabetics with infection in our study were able to increase their neutrophil counts as well as the respiratory burst activity (higher NBT score).

Irrespective of the state of diabetes, our study confers that mean NBT and ANC were significantly higher in patients with infection.

Comparison with a standard study between non-diabetics (c) and diabetics (d) without infection:

On comparing the mean NBT scores of the diabetics and the non-diabetics without infection categories of our study with that of other standard studies, our results ,i.e., a higher basal mean NBT score in diabetics,compared well with those of Kruszewski *et al*[43] in 1979 , Lechowski *et al*[44] in 1991 and Larijani *et al*[46] in 2007 but not with Yun Woong Ko *et al*[74] which might be due to a small sample size.

Table 22 Comparison of NBT scores and ANC

		Yun Woong Ko *et al* 1977	**Present study**
Non-diabetics without infection (c)	No. of cases	27	31
	Mean NBT	3.2	21.8
	Mean ANC	4	4.3
Diabetics without infection (d)	No. of cases	10	33
	Mean NBT	2.6	32.7
	Mean ANC	3.5	4.6

The higher mean NBT score in diabetics (d) compared to the non-diabetics without infection (c) could be related to the persistent activated state of neutrophils seen in diabetics due to AGEs[42]. Nuran Nabi *et al*[45] in 2005 , also found that diabetic rats showed higher NBT scores compared to control healthy rats ($P<0.001$) which was comparable to other neutrophil function tests like polarization assay where the neutrophils of diabetic rats were more polarized at baseline level compared to control rats.

Comparison with standard study between non-diabetics (i) and diabetics (di) with infection

Table 23 Comparison of NBT scores and ANC

		Yun Woong Ko *et al* 1977	**Present study**
Non-diabetics with Infection (i)	No. of cases	10	31
	Mean NBT %	10.6	76.5
	Mean ANC $x10^9$/L	10.4	6.1
Diabetes with infection (di)	No. of cases	10	32
	Mean NBT%	4.6	63.4
	Mean ANC $x10^9$/L	9.9	7.6

Despite the presence or absence of diabetes, both the groups with infection (di, i) had significantly high NBT scores and mean ANCs compared to their non-infected counterparts(d,c) respectively, but the mean NBT scores of diabetics with infection(63.4%) did not reach the high mean NBT scores of non-diabetics with infection(76.5%). This difference was significant ($P<0.05$).

Our results were in concurrence with the standard study by Larijani *et al*[46] in 2007, who compared 19 diabetic foot patients with 20 controls. The mean NBT scores of the non-infected diabetics in their study was significantly higher compared to controls, whereas stimulated NBT test showed an inadequate rise in diabetics but a marked rise in controls.

It could probably be related to the fact that the persistent hyper-excited state of neutrophils in diabetics leads to 'burn-out' of neutrophils which respond inadequately when stimulated by an infectious pathogen. This burnt-out state had been demonstrated by Shah *et al,*[37] who showed reduced superoxide radical production by neutrophils of diabetics on exposure to infecting pathogen. The production of superoxide and other free radicals require NADPH-oxidase which is also essential for NBT reduction. Hence the constant state of activation of neutrophils in resting state in diabetics could probably also result in loss of enzymes necessary for oxygen derived free radical production, when stimulated by infectious pathogens leading to a inadequate functional response (NBT score).[46]

Binding of advanced glycation end products (AGEs) to lysosomal enzymes as was demonstrated by Li.Y.M[41] could also play a role in the reduced elevation of respiratory burst.

Comparing the two NBT scoring systems

Table 24 : Percentage of case distribution in 1st (classic) scoring system

S.No.	NBT score range	c (%)	i (%)	d (%)	di (%)
1	0-20	61.3	0	24.2	3.1
2	21-40	29	6.4	54.5	18.8
3	41-60	6.5	12.9	9.1	25
4	61-80	3.2	35.5	6.1	18.7
5	81-100	0	45.2	6.1	34.4
	Total %	100	100	100	100

Table 25 : Percentage of case distribution in 2nd (Grading) system

S.No.	NBT score range	c (%)	i (%)	d (%)	di (%)
1	0-50	39.3	3.3	0	0
2	51-100	25	3.3	45.5	0
3	101-150	25	6.7	33.3	32.3
4	151-200	10.7	16.7	12.1	22.6
5	201-250	0	30	6.1	22.6
6	251-300	0	26.7	3	16.1
7	301-350	0	13.3	0	6.4
8	351-400	0	0	0	0
	Total %	100	100	100	100

Figure 13 : Percentage case distribution (1st scoring system)

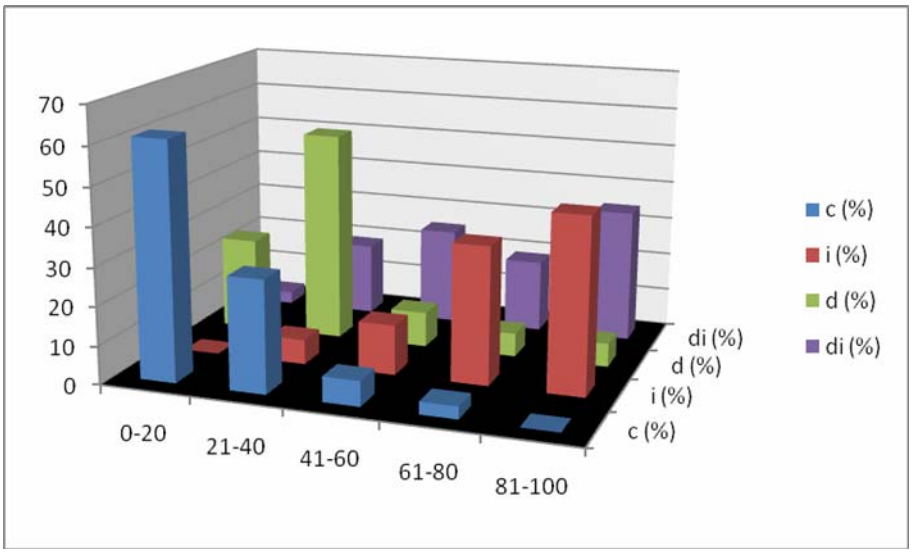


Figure 14: Percentage case distribution (2nd scoring system)

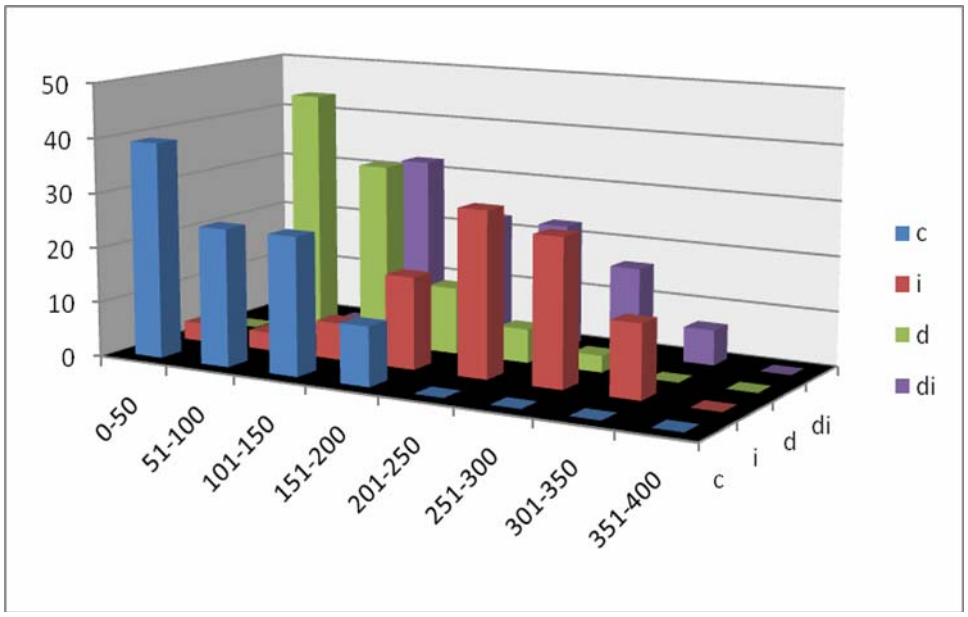


Table 26 : Comparison of the two scoring systems based on the difference in proportion of cases

c Vs d	Score range	Categories compared	Standard Error of difference between 2 proportions	Z	Significance
Classic system	0-20	c>d	11.49	3.23	P<0.05
	21-40	d>c	11.89	2.14	P<0.05
Grading system	0-50	c>d	9.23	4.25	P<0.05
	51-150	d>c	11.82	2.43	P<0.05
i Vs di	Score range				
Classic system	21-40	di>i	8.18	1.51	P>0.05
	61-100	i>di	11.31	2.43	P<0.05
Grading system	51-150	di>i	10.03	2.22	P<0.05
	201-400	i>di	12.24	2.03	P<0.05

Comparing the two scoring systems for non-diabetics (c) and diabetics 9d) without infection

It was observed from the analysis tabulated above (tables 24,25,26) that in the lowest score range in both systems (i.e.,0-20 in the 1st and 0-50 in 2nd), the proportion of cases of non-diabetics without infection (c) was more than

that of diabetics without infection (d) category (P<0.05), whereas in the low middle score ranges (i.e., 21-40 in the 1st and 51-150 in the 2nd), the proportion of cases of diabetes without infection (d) was higher than that of non-diabetics without infection (c) category (P<0.05). Moreover, with reference to table no. 25, in the grading system, 45.3% of patients in the diabetes without infection (d) category had score range of 51-100, while none in this category's (d) cases were in the 0-50 range indicating that none of the diabetics in our study had inactive neutrophils. Whereas in the classical (1st) scoring system (table 24), although 54.5% of patients of (d) category were in the 21-40 range, there was also a sizeable representation in the 0-20 range i.e.,24.2%. Hence it was inferred that even though both the scoring systems were able to highlight the higher neutrophil activity of diabetics compared to non-diabetics without infection, the same could be better appreciated by the grading system.

Comparing the two scoring systems for Non-diabetics (i) and diabetics (di) with infection

It was also observed (from the table 24,25 and 26) that in the higher score range in both systems (i.e., 61-100 in the 1st and 200-400 in the 2nd), the proportion of cases of diabetics with infection (di) was lower than that of non-diabetics with infection (i) category (P<0.05), whereas in the low middle score range (i.e., 21-40 in the 1st and 51-150 in the 2nd system), the proportion of cases of diabetics with infection (di) was found to be higher than that of non-diabetics with infection (i) category, but this difference was statistically

significant in the grading system but not in the classic system. Hence it was inferred that both the scoring systems showed lower rise in NBT scores in diabetics with infection (di) compared to non-diabetics with infection (i) probably reflecting a reduced neutrophil activity in diabetics with infection, though it was better brought out by the grading system.

SUMMARY AND CONCLUSION

- This study has attempted to analyze the bactericidal function of the neutrophils by the simple NBT test in diabetes mellitus.
- This study has employed both the classical NBT scoring system (based on the presence or absence of formazan granules in cytoplasm) and the 2nd scoring system (based on the grading of staining).
- The study population included non-diabetics without infection , non-diabetics with infection, diabetics without infection and diabetics with infection.

Figure 15

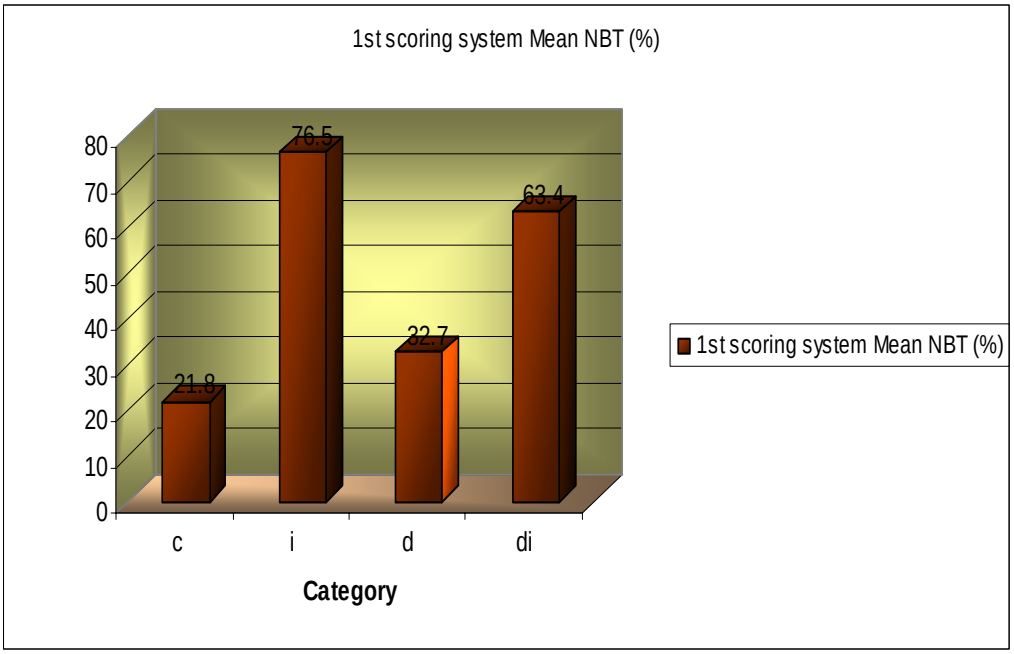
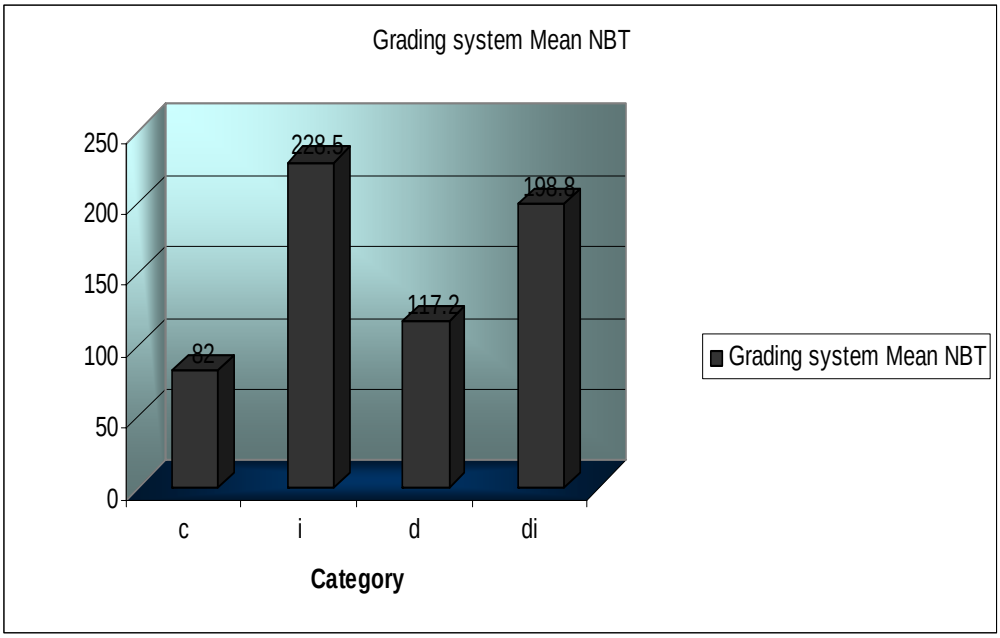


Figure 16



- In this study, higher NBT scores were observed in diabetics when compared to non-diabetics without infection. It could probably be as a result of persistent activation of resting neutrophils in diabetics compared to non-diabetics :: [1st scoring system : non-diabetics without infection (mean NBT=21.8%); diabetics without infection (mean NBT=32.7%). 2nd scoring system: non-diabetics without infection (mean NBT=82/400); diabetics without infection (mean NBT=117.2/400)]. However there was no significant difference in the ANCs of diabetics and non-diabetics without infection.
- Irrespective of the diabetic status, patients when exposed to infectious agents, had higher mean NBT scores and absolute neutrophil counts, thereby confirming NBT test as a useful test to ascertain the presence of infection :: [1st scoring system : non-diabetics with infection (mean NBT=76.5%); diabetics with infection (mean NBT=63.4%). 2nd scoring

system : non-diabetics with infection (mean NBT=228.5/400); diabetics with infection (mean NBT=198.8/400)]

- However, the mean NBT score of diabetics with infection (63.4%) did not reach the higher mean NBT scores of non-diabetics with infection (76.5%), probably related to the hyper-excited state of resting neutrophils, leading to burn out and reduced ability of neutrophils to mount a high respiratory burst activity (defective bactericidal function). And this difference has been better highlighted by the grading system.
- No correlation was found between NBT scores and gender in our study.
- There was a positive correlation between NBT scores and absolute neutrophil counts in non-diabetics without infection, non-diabetics with infection and diabetics without infection. However, a negative correlation was observed in diabetics with infection probably also indicating a fall in neutrophil function in that population.

Pitfalls in our study:

- Only patients who visited our hospital were chosen for our study. Selection was not extended to a wider area.

- The relationship between duration of diabetes mellitus and NBT scores was not analyzed. Only a single blood sugar level evaluated.

- The degree of control of diabetes mellitus (HbA1c) was not evaluated.

- This study is only a single point analysis. Serial evaluation of subjects has not been done.

Prospects of Future Studies:

1. To analyse if NBT test correlates with the severity of diabetes mellitus (HbA1c levels).
2. The feasibility of NBT scoring as a screening test of neutrophil function, while evaluating patients with diabetes mellitus, and its predictive value in response to infections.
3. In view of the low cost of NBT scoring and its technical simplicity, the same may be compared to other tests of neutrophil function and the scoring standardized.
4. To in-cooperate grading in NBT scoring to effect a standardized evaluation and eliminate inter-observer variation observed in the classical scoring.
5. To re-inforce our observation of increased sensitivity by the graded system of scoring by studies with a larger sample size.

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Y

Master Chart

S.No.	*Slide No.*	Ip/Op No.	Age	Sex	Fever	Antibiotic	Antibiotic(days)	DM	DM Duration(years)	O/I/B/N	Diagnosis	TLC(X10^9/L)	P (%)	L(%)	E(%)	ESR(mm/hr)	FBS (mg/dl)	Culture	Others	Organism	ANC(X10^9/L)	NBT score(%)	NBT Grading score/400
1	***i02***	84802/07	43	M	Y	Y	3	N	NA	NA	Ulcers foot	14.8	86	7	7	36	82	Pus		Pseudomanas	12.7	97	
2	***i09***	25012/08	22	F	Y	N	0	N	NA	NA	Enteric fever	3.8	49	45	6	72	80		WIDAL	S.typhi	1.9	72	291
3	***i15***	26122/08	23	F	Y	Y	2	N	NA	NA	Meningo encephalitis	3.4	69	29	2	24	117		WIDAL	S.typhi	2.4	88	75
4	***i16***	26766/08	19	F	Y	Y	2	N	NA	NA	Vaginitis	6.8	60	37	3	26	46	Pus		Staph.aureus	4.1	89	348
5	***i17***	25657/08	30	M	Y	Y	1	N	NA	NA	Leptospirosis	4.4	50	42	8	20	95		MSAT	Leptospira	2.2	93	190
6	***i19***	26972/08	35	F	Y	N	0	N	NA	NA	Leptospirosis	8.2	50	48	2	24	94		MSAT	Leptospira	4.1	70	127
7	***i20***	26864/08	75	M	Y	Y	3	N	NA	NA	Ulcer foot	8.5	43	42	15	25	70	Pus		Klebsiella	3.7	94	214
8	***i22***	49709/08	73	M	N	Y	7	N	NA	NA	Ulcer foot	10	69	30	1	15	80	Pus		E.coli	6.9	48	177
9	***i23***	50250/08	68	F	N	Y	7	N	NA	NA	UTI	7	52	40	8	70	90	Urine		E.coli	3.6	26	48
10	***i24***	41832/08	43	M	N	Y	10	N	NA	NA	Septicemia	7.9	47	49	4	5	98	Blood		Pseudomanas	3.7	68	176
11	***i25***	53373/08	30	M	N	Y	3	N	NA	NA	Wound infection	10	89	10	1	7	140	Pus		Klebsiella	8.9	55	191
12	***i27***	51460/08	30	M	Y	N	0	N	NA	NA	Abscess palm	11.3	80	12	8	36	106	Pus		Pseudomanas	9.0	96	277
13	***i30***	52231/08	31	M	N	Y	8	N	NA	NA	Fournier's gangrene	9.1	68	30	2	15	108	Pus		Pseudomanas	6.2	81	155
14	***i31***	53809/08	60	M	N	Y	3	N	NA	NA	Necrotising fasciitis	10.5	80	16	4	38	100	Pus		Acinetobacter	8.4	87	270
15	***i32***	45725/08	30	M	N	Y	14	N	NA	NA	Gluteal abscess	11	80	15	5	24	74	Pus		E.coli	8.8	80	292
16	***i33***	59193/08	22	M	N	Y	60	N	NA	NA	Wound infection	8.5	58	38	4	20	90	Pus		Klebsiella	4.9	75	308
17	***i34***	74514/08	28	F	Y	Y	1	N	NA	NA	Enteric fever	11.2	60	37	3		85		WIDAL	S.typhi	6.7	91	245
18	***i35***	72617/08	22	M	N	Y	5	N	NA	NA	Wound infection	9.9	62	31	4	37	113	Pus		Proteus	6.1	74	275
19	***i36***	68545/08	34	M	Y	Y	4	N	NA	NA	Wound infection	6.8	65	31	4	16	151	Bile		Pseudomanas	4.4	75	231
20	***i37***	71045/08	40	M	Y	N	0	N	NA	NA	UTI	6.2	90	8	2	3	117	Urine		Acinetobacter	5.6	95	229
21	***i38***	71377/08	29	M	Y	Y	6	N	NA	NA	Wound infection	30.9	86	7	7	86	77	Pus		Klebsiella	26.6	96	343
22	***i39***	70291/08	38	M	N	Y	10	N	NA	NA	Wound infection	7.3	69	29	2	45	78	Pus		Pseudomanas	5.0	72	273
23	***i40***	74124/08	48	M	Y	N	0	N	NA	NA	Lung cavity	8.9	78	17	5	34	60	Sputum		Klebsiella	6.9	72	203
24	***i41***	74328/08	37	F	Y	N	0	N	NA	NA	Pneumonia	9.1	71	27	2	22	97	Sputum		Klebsiella	6.5	90	246
25	***i42***	72067/08	26	M	N	N	0	N	NA	NA	Wound infection	7.6	65	31	4	10	69	Pus		CONS	4.9	54	136
26	***i43***	72948/08	47	M	N	Y	4	N	NA	NA	Abscess thigh	3.4	41	55	5	28	64	Pus		E.coli	1.4	92	340
27	***i45***	75354/08	54	M	N	Y	10	N	NA	NA	Wound infection	7.2	62	32	6	12	109	Pus		Proteus	4.5	85	
28	***i46***	74931/08	27	M	N	N	0	N	NA	NA	Wound infection	8.2	65	34	1	18	94	Pus		Klebsiella	5.3	80	209
29	***i47***	74380/08	65	M	N	N	0	N	NA	NA	Pneumonia	6.4	54	40	6	11	157	Sputum		Pseudomanas	3.5	40	215
30	***i48***	48327/08	38	M	Y	Y	120	N	NA	NA	Wound infection	7.2	60	32	8	21	90	Pus		Proteus	4.3	76	270
31	***i49***	76775/08	24	M	N	Y	5	N	NA	NA	Wound infection	8.6	58	40	2	6	85	Pus		Proteus	5.0	60	385

S.No.	*Slide No.*	Ip/Op No.	Age	Sex	Fever	Antibiotic	Antibiotic(days)	DM	DM Duration(years)	O/I/B/N	Diagnosis	TLC(x10^9/L)	P(%)	L(%)	E(%)	ESR(mm/hr)	FBS mg/dl	Culture	Others	Organism	ANC(x10^9/L)	NBT score(%)	NBT Grading score /400
32	***di01***	84924/07	67	M	Y	Y	1	Y	3	O	Ulcer foot	12	84	13	3	43	165	Pus		Proteus	10.1	56	121
33	***di02***	167767/07	85	F	Y	Y	4	Y	4	O	Osteomyelitis	8.4	67	28	5	55	140	Pus		Staph.aureus	5.6	36	129
34	***di03***	21450/08	33	M	Y	Y	18	Y	2	O	Melioidosis	16.6	89	8	1	34	171	Blood		B.pseudomallei	14.7	63	243
35	***di04***	22542/08	46	M	N	N	0	Y	2	O	Peritonitis	8.4	63	34	3	15	150	Ascites		CONS	5.3	74	225
36	***di05***	70022/08	70	M	N	Y	2	Y	3	O	UTI	10.2	65	28	7	7	294	Urine		Pseudomanas	6.6	84	199
37	***di08***	23728/08	50	M	N	Y	2	Y	10	I	Wound infection	8.2	82	16	2	10	139	Pus		Proteus	6.7	77	300
38	***di11***	50426/08	27	M	N	Y	6	Y	7	I	UTI	9	64	34	2	15	64	Urine		E.coli	5.8	26	239
39	***di13***	53490/08	60	M	Y	Y	5	Y	3	O	Ulcer foot	9.4	75	20	5	28	270	Pus		Proteus	7.1	54	187
40	***di14***	46170/08	58	F	N	Y	30	Y	10	O	Ulcer foot	8.4	60	30	4	35	212	Pus		Pseudomanas	5.0	63	
41	***di15***	53658/08	45	F	N	Y	5	Y	10	O	Ulcer foot	17.3	88	6	6	30	248	Pus		Klebsiella	15.2	74	311
42	***di16***	72745/08	20	M	Y	Y	2	Y	2	I	Enteric fever	8.6	65	33	2	25	216		WIDAL	S.typhi	5.6	56	118
43	***di17***	55679/08	49	M	N	Y	6	Y	4	O	Ulcer foot	6.6	62	37	1	42	328	Pus		Klebsiella	4.1	88	262
44	***di18***	64742/08	45	F	N	N	0	Y	7	O	Ulcer foot	13.1	87	10	3	33	126	Pus		CONS	11.4	84	294
45	***di19***	67402/08	44	M	N	Y	3	Y	4	O	Ulcer foot	10.2	52	38	5	10	198	Pus		Klebsiella	5.3	90	223
46	***di20***	68202/08	55	M	N	Y	1	Y	2	O	Ulcer foot	29.1	93	5	2	40	500	Pus		CONS	27.1	28	112
47	***di21***	67136/08	60	F	Y	Y	6	Y	15	O	Ulcer foot	12.1	80	16	4	22	142	Pus		Pseudomanas	9.7	77	215
48	***di22***	70838/08	60	M	Y	N	0	Y	10	O	Ulcer foot	9.5	70	25	5	36	84	Pus		Proteus	6.7	20	116
49	***di24***	67494/08	38	M	N	Y	2	Y	2	O	Ulcer foot	17.5	70	28	2	25	134	Pus		Proteus	12.3	32	150
50	***di25***	71172/08	42	F	N	Y	5	Y	7	O	Ulcer foot	8.7	59	33	8	14	162	Pus		Klebsiella	5.1	26	
51	***di27***	68192/08	40	F	N	N	0	Y	4	O	Asymptomatic UTI	7.4	66	33	1	15	173	Urine		E.coli	4.9	54	165
52	***di28***	30773/98	57	F	N	N	0	Y	10	I	Asymptomatic UTI	8.7	74	23	3	21	126	Urine		Citrobacter	6.4	87	309
53	***di29***	73364/08	60	F	Y	Y	4	Y	4	O	Acute Pyelonephritis	10.4	90	8	2	46	252	Urine		E.coli	9.4	89	289
54	***di30***	74429/08	50	M	Y	Y	4	Y	2	O	UTI	6.9	85	12	3	40	105	Urine		E.coli	5.9	54	129
55	***di32***	73127/08	66	M	N	N	0	Y	2	O	UTI	9.4	83	14	3	60	162	Urine		Klebsiella	7.8	40	106
56	***di33***	73238/08	60	M	N	Y	10	Y	10	O	Ulcer scrotum	8.4	52	40	8	40	199	Pus		Pseudomanas	4.4	87	225
57	***di34***	60840/08	55	M	Y	Y	20	Y	5	O	UTI	4	50	45	5	124	108	Urine		Klebsiella	2.0	82	149
58	***di35***	75249/08	46	F	Y	Y	5	Y	12	O	UTI	8.8	64	34	2	35	270	Urine		E.coli	5.6	56	184
59	***di36***	72432/08	60	F	N	N	0	Y	8	O	Bed sore	10	89	9	2	41	154	Pus		Proteus	8.9	88	251
60	***di37***	72723/08	58	F	Y	Y	5	Y	5	B	Ulcer foot	9.6	72	26	2	42	228	Pus		Proteus	6.9	84	173
61	***di38***	75463/08	50	F	N	Y	10	Y	6	B	Ulcer foot	7	73	22	5	80	216	Pus		Proteus	5.1	90	193
62	***di39***	42346/00	59	M	N	N	0	Y	2		Asymptomatic UTI	6.5	64	28	4	23	211	Urine		E.coli	4.2	52	117
63	***di41***	29292/97	50	M	N	N	0	Y	2		Asymptomatic UTI	5.8	59	39	2	13	196	Urine		CONS	3.4	57	193

	Slide No.	Ip/Op No.	Age	Sex	Fever	Antibiotic(days)	DM	DM Duration(years)	O/I/B/N	Diagnosis	TLC(x10^9/L)	P(%)	L(%)	E(%)	ESR(mm/hr)	FBS (mg/dl)	Culture	Others	Organism	ANC (x10^9/L)	NBT score(%)	NBT Grading score/400
64	***d01***	22187/08	38	M	N	N	Y	2	I	DKA	8.7	70	26	4	12	400	Urine		NG	6.1	23	104
65	***d02***	24425/08	74	M	N	N	Y	15	I	DM Nephropathy	7.4	60	36	4	40	200	Urine		NG	4.4	6	175
66	***d03***	22628/08	27	M	N	N	Y	2	B	DKA	8.7	62	36	2	35	300	Urine		NG	5.4	28	55
67	***d04***	22886/08	40	M	N	N	Y	2	O	DM Neuropathy	8.7	65	33	2	25	276	Urine		NG	5.7	37	184
68	***d05***	24429/08	40	F	N	N	Y	6	B	DM	8.2	68	30	2	7	370	Urine		NG	5.6	23	123
69	***d06***	23463/08	75	F	N	N	Y	3	O	DM Neuropathy	7.6	62	35	3	25	166	Urine		NG	4.7	28	66
70	***d07***	25659/08	78	F	N	N	Y	15	O	DM	9.6	55	43	2	25	218	Urine		NG	5.3	56	227
71	***d10***	50608/02	59	M	N	N	Y	6	O	DM	7.2	54	44	2	8	168	Urine		NG	3.9	27	117
72	***d12***	61733/08	31	F	N	N	Y	8	O	DM	6.1	72	27	1	24	179	Urine		NG	4.4	23	132
73	***d13***	15746	65	M	N	N	Y	15	O	DM	8.2	68	28	4	12	188	Urine		NG	5.6	68	189
74	***d14***	43515/01	40	F	N	N	Y	5	O	DM	5.4	50	46	4	8	202	Urine		NG	2.7	19	71
75	***d15***	149376/07	48	F	N	N	Y	7	O	DM	8.1	54	42	4	10	180	Urine		NG	4.4	20	54
76	***d16***	67801/08	52	M	N	N	Y	4	O	DM	6.2	68	30	2	12	144	Urine		NG	4.2	9	95
77	***d17***	3560	49	M	N	N	Y	3	O	DM	6.6	72	24	4	22	225	Urine		NG	4.8	23	83
78	***d18***	1882260	49	F	N	N	Y	7	O	DM	8.8	70	28	2	18	228	Urine		NG	6.2	66	156
79	***d20***	537907	45	F	N	N	Y	5	B	DM	9.2	66	33	1	8	105	Urine		NG	6.1	58	136
80	***d21***	10296	48	F	N	N	Y	20	B	DM	10.8	72	26	2	10	126	Urine		NG	7.8	91	244
81	***d22***	1973	45	F	N	N	Y	4	O	DM	7.8	62	34	4	14	202	Urine		NG	4.8	58	138
82	***d24***	83407	49	M	N	N	Y	3	O	DM	9.5	60	34	6	12	165	Urine		NG	5.7	28	260
83	***d25***	404040	55	M	N	N	Y	4	O	DM	5.4	52	46	2	26	192	Urine		NG	2.8	25	102
84	***d26***	61868/05	55	M	N	N	Y	3	O	DM	6.8	66	33	1	8	164	Urine		NG	4.5	17	60
85	***d27***	68253/08	58	M	N	N	Y	5	O	DM	7.6	58	38	1	6	102	Urine		NG	4.4	32	89
86	***d28***	57540/04	60	F	N	N	Y	9	O	DM	8.7	70	22	8	10	112	Urine		NG	6.1	22	147
87	***d29***	142014	53	F	N	N	Y	14	O	DM	9.6	68	30	1	16	160	Urine		NG	6.5	85	140
88	***d30***	17716/90	63	M	N	N	Y	18	O	DM	5.6	54	35	1	8	186	Urine		NG	3.0	15	110
89	***d31***	11101	68	F	N	N	Y	10	O	DM	7.2	64	32	2	12	143	Urine		NG	4.6	21	58
90	***d32***	53739/05	50	F	N	N	Y	5	O	DM	6.4	56	40	4	10	154	Urine		NG	3.6	22	51
91	***d33***	38957/99	50	M	N	N	Y	9	O	DM	3.6	28	60	12	20	189	Urine		NG	1.0	25	56
92	***d34***	54740/03	50	F	N	N	Y	5	O	DM	2.7	48	48	4	18	124	Urine		NG	1.3	21	106
93	***d35***	1636/01	62	M	N	N	Y	8	O	DM	9.2	56	40	4	11	160	Urine		NG	5.2	35	86
94	***d36***	94810	59	F	N	N	Y	3	O	DM	6.1	64	32	4	13	155	Urine		NG	3.9	33	100
95	***d37***	29826/97	60	F	N	N	Y	11	O	DM	8	68	24	8	5	110	Urine		NG	5.4	17	81
96	***d38***	79018	62	M	N	N	Y	2	O	DM with CVA	3.7	48	36	16	14	200	Urine		NG	1.8	19	72

S.No.	*Slide No.*	Age	Sex	Fever	Antibiotic(days)	DM	DM Duration(years)	OI/IB/N	Diagnosis	TLC($x10^9$/L)	P(%)	L(%)	E(%)	ESR(mm/hr)	FBS (mg/dl)	Culture	Others	Organism	ANC($x10^9$/L)	NBT score(%)	NBT Grading score/400
97	***c01***	33	F	N	N	N	NA	NA	Volunteer	8.5	52	46	2	8	84	NA	NA	NA	4.4	35	50
98	***c02***	29	M	N	N	N	NA	NA	Volunteer	5.4	59	40	1	6	80	NA	NA	NA	3.2	20	56
99	***c03***	28	M	N	N	N	NA	NA	Volunteer	9.2	62	38	0	6	105	NA	NA	NA	5.7	43	22
100	***c04***	30	M	N	N	N	NA	NA	Volunteer	7.2	58	29	8	10	94	NA	NA	NA	4.2	16	36
101	***c05***	23	M	N	N	N	NA	NA	Volunteer	7.6	62	36	2	12	84	NA	NA	NA	4.7	14	72
102	***c07***	19	M	N	N	N	NA	NA	Volunteer	6.9	57	43	0	6	102	NA	NA	NA	3.9	22	84
103	***c08***	19	M	N	N	N	NA	NA	Volunteer	5.2	72	25	3	8	90	NA	NA	NA	3.7	10	72
104	***c09***	19	M	N	N	N	NA	NA	Volunteer	8.1	71	22	7	6	88	NA	NA	NA	5.7	10	132
105	***c10***	19	M	N	N	N	NA	NA	Volunteer	6.9	58	33	9	8	102	NA	NA	NA	4	11	50
106	***c11***	19	M	N	N	N	NA	NA	Volunteer	9.6	70	26	4	10	90	NA	NA	NA	6.7	17	79
107	***c12***	19	M	N	N	N	NA	NA	Volunteer	7.6	72	26	2	6	92	NA	NA	NA	5.5	19	43
108	***c13***	19	M	N	N	N	NA	NA	Volunteer	3.8	34	40	12	15	96	NA	NA	NA	1.2	14	129
109	***c15***	20	M	N	N	N	NA	NA	Volunteer	6	52	45	3	20	106	NA	NA	NA	3.1	20	13
110	***c16***	20	M	N	N	N	NA	NA	Volunteer	8	48	40	8	12	84	NA	NA	NA	3.8	60	38
111	***c17***	19	F	N	N	N	NA	NA	Volunteer	9	80	19	1	6	92	NA	NA	NA	7.2	21	21
112	***c18***	20	F	N	N	N	NA	NA	Volunteer	7.5	84	12	1	8	100	NA	NA	NA	6.3	15	38
113	***c19***	19	F	N	N	N	NA	NA	Volunteer	9.6	60	35	3	6	84	NA	NA	NA	5.7	18	34
114	***c20***	19	F	N	N	N	NA	NA	Volunteer	8.4	88	11	1	8	96	NA	NA	NA	7.4	19	12
115	***c22***	25	F	N	N	N	NA	NA	Volunteer	10.6	76	20	2	14	98	NA	NA	NA	8.1	20	161
116	***c23***	18	F	N	N	N	NA	NA	Volunteer	2.8	32	60	8	6	86	NA	NA	NA	0.9	27	114
117	***c27***	18	F	N	N	N	NA	NA	Volunteer	5.2	60	32	8	6	84	NA	NA	NA	3.1	24	141
118	***c28***	32	F	N	N	N	NA	NA	Volunteer	5.8	68	24	4	8	112	NA	NA	NA	3.9	8	
119	***c29***	20	F	N	N	N	NA	NA	Volunteer	6.3	68	24	2	12	96	NA	NA	NA	4.3	22	147
120	***c30***	24	M	N	N	N	NA	NA	Volunteer	10.1	60	36	2	10	106	NA	NA	NA	6.1	35	152
121	***c31***	19	F	N	N	N	NA	NA	Volunteer	3.5	56	40	4	6	86	NA	NA	NA	2	17	
122	***c33***	41	M	N	N	N	NA	NA	Volunteer	3.1	72	26	2	14	118	NA	NA	NA	2.2	31	142
123	***c34***	20	F	N	N	N	NA	NA	Volunteer	3.5	44	40	12	6	86	NA	NA	NA	1.5	8	167
124	***c35***	34	F	N	N	N	NA	NA	Volunteer	5.1	60	32	4	8	94	NA	NA	NA	3.1	24	116
125	***c36***	28	M	N	N	N	NA	NA	Volunteer	2.8	58	40	2	10	102	NA	NA	NA	1.6	4	90
126	***C37***	30	F	N	N	N	NA	NA	Volunteer	6.2	70	28	2	6	90	NA	NA	NA	4.3	9	85
127	***c38***	20	M	N	N	N	NA	NA	Volunteer	8.6	72	20	8	6	88	NA	NA	NA	6.2	65	

Key to Master Chart

B	-	On Both oral hypoglycemics and insulin
DKA	-	Diabetic Keto Acidosis
DM	-	Diabetes Mellitus
E	-	Eosinophils
ESR	-	Erythrocyte Sedimentation Rate
FBS	-	Fasting Blood Sugar
I	-	On Insulin
L	-	Lymphocytes
N	-	No / Not on treatment
NA	-	Not Applicable
NG	-	No Growth
O	-	On Oral hypoglycemic drug
P	-	Polymorphs
TLC	-	Total Leucocyte Count
Y	-	Yes

ANNEXURES

INSTITUTIONAL ETHICAL COMMITTEE
GOVERNMENT GENERAL HOSPITAL & MADRAS MEDICAL COLLEGE,
CHENNAI-600 003.

Telephone: 044-2530 5000

Fax : 044 – 25305115

K.Dis.No.16328 P & D3/Ethies/Dean/GGH/08 Dated 8/9/2008

Title of the work : "Utility of Nitro Blue Tetrazolium Test (NBT) to assess neutrophil function in blood samples in diabetics & non diabetics with or without infection"

Principal Investigator :

Department : Dr. Jonathan Arnold A.P., Pathology, GH & MMC, Ch-3.

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 10th Sep 2008 at 2 P.M in , Deans, Chamber, Chennai-3.

The members of the Committee, the Secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The principal investigator and their term are directed to adhere the guidelines given below:

1. You should get detailed informed consent from the patients/participants and maintain confidentiality.
2. You should carry out the work without detrimental to regular activities as well as without extra expenditure to the Institution or Government.
3. You should inform the IEC in case of any change of study procedure, site and investigation or guide.
4. You should not deviate form the area of the work for which I applied for ethical clearance.
5. You should inform the IEC immediately, in case of any adverse events or serious adverse reactions.
6. You should abide to the rules and regulations of the institution(s)
7. You should complete the work within the specific period and if any extension of time is required, you should apply for permission again and do the work.
8. You should submit the summary of the work to the ethical committee on completion of the work.
9. You should not claim funds from the Institution while doing the work or on completion.
10. You should understand that the members of IEC have the right to monitor the work with prior intimation.

SECRETARY
IEC, GGH,CHENNAI

CHAIRMAN
IEC, GGH,CHENNAI

DEAN
GGH &MMC, CHENNAI

RKM.5.6(2)

PROFORMA

Slide No: _____

Name: Age: Sex:

IP NO: Unit/Ward

Occupation: Address:

History:

Fever: Yes _____ No_____ Duration:

Diabetes: Duration Type: I ___ II. _____

Oral Hypoglycemics

Insulin

Antibiotic: Duration:

Other Treatments:

General Examination:

Skin / subcutaneous

Nails:

Foot:

Eye:

NT:

Oral cavity:

CVS:

RS:

CNS:

P/A:

Perineum/Ext. Gen

Investigations:

TLC: FBS:

DLC:

ESR: mm/hr Urine Routine:

Culture

Other Investigations:

Summary:

Diabetes: Yes___ No____

Infection: Yes ____ No _____ Diagnosis / Type of Infection: _____

NBT Score:

A. Classical scoring system:
B. Grading system: